## REMARKS

Entry of the foregoing, reexamination and reconsideration of the subject application, as amended, pursuant to and consistent with 37 C.F.R. § 1.112, are respectfully requested in light of the remarks which follow.

### I. Amendments to the Specification

By the foregoing amendments to the specification, the sequence identifiers have been inserted (see below for more detail).

### II. Amendments to the Claims

By the foregoing amendments to the claims, claims 1-4, 6-11, 13, 14, 16-20, and 57-61 have been amended, new claims 62-65 have been added, and claim 21 has been canceled.

In particular, claims 1-4 have been amended to clarify that the oocytes, blastocyts, and resultant stem cell lines are human.

Claims 16, 18, and 19 have been amended to clarify that the feeder cells of at least one of step ii) and step iv) are embryonic, of mouse or human origin, or mitotically inactivated, respectively.

Claims 4, 9, 60, and 61 have been amended by deleting the word "about."

Other amendments have been made to the claims to clarify the claim language. These amendments are merely editorial in nature and are not intended to change the scope of the claims or elements recited therein.

New claims 62-65 recite that the feeder cells of step ii) and step iv) are human or mouse embryonic feeder cells, or more particularly human or mouse embryonic fibroblasts. Claims 62-65 are supported in at least at page 7, lines 4-8 and in the examples (*see, e.g.,* Example 4 at page 17).

The amendments to the claims, including cancellation of claims, have been made without prejudice or disclaimer to any subject matter recited or canceled herein. Applicant reserves the right to file one or more continuation and/or divisional applications directed to any canceled subject matter. No new matter has been added, and entry of the foregoing amendments of the above-identified application is respectfully requested.

## III. Response to Objection to the Sequence Listing

The Examiner has stated that the present application fails to comply with the Sequence Listing requirements under 37 C.F.R. §§ 1.821(a)(1) and (a)(2).

Applicant submits herewith a Sequence Listing that contains all of the sequences set forth in the specification that are encompassed by the sequence definitions set forth in 37 C.F.R. §§ 1.821(a)(1) and (a)(2). In particular, Applicant submits herewith a computer readable form (CRF) copy of the Sequence Listing, a paper copy of the Sequence Listing, and the required statement to support the Sequence Listing. In addition, Applicant has amended the specification to identify each sequence with the corresponding sequence identification number.

In view of the above, Applicant respectfully requests reconsideration and withdrawal of this objection.

## IV. Response to Objection to the Claims

Claim 17 has been objected to because it does not have an appropriate article before the term "animal source."

Applicant has amended claim 17 to recite "*an* animal source," as suggested by the Examiner.

Accordingly, Applicant respectfully requests reconsideration and withdrawal of this objection.

## V. Response to Claim Rejections Under 35 U.S.C. § 112, First Paragraph

Claims 1-21, 35, and 57-61 have been rejected under 35 U.S.C. § 112, first paragraph, as purportedly lacking enablement.

Specifically, the Examiner has acknowledged that the specification enables obtaining pluripotent human blastocyst-derived stem cells wherein the inner cell mass cells are co-cultured on fibroblast feeder cells, and the blastocyst-derived stem cell line is propagated in fibroblast feeder cells. However, it is the Examiner's position that the specification does not enable culturing inner cell mass cells or blastocyst-derived stem cell lines on feeder cells other than fibroblast feeder cells.

In support of this position, the Examiner has stated that the working examples in the specification are directed to culturing human blastocyst-derived stem cell lines on embryonic

mouse fibroblasts, and does not provide guidance with regard to other types of feeder cells. The Examiner has further stated that the art teaches that human embryonic stem cells require fibroblast feeder cells, and that alternative effective methods of culturing human embryonic stem cells are not known. This rejection is respectfully traversed, for at least the following reasons:

Applicant submits that a person of ordinary skill in the art would have reasonably predicted that additional feeder cells within the scope of the claims are likely to be effective in propagating pluripotent human blastocyst-derived stem cell lines. As stated in *Capon v. Eshhar*, 418 F.3d 1349, 1359, (Fed. Cir. 2005), "it is not necessary that every permutation within a generally operable invention be effective in order for an inventor to obtain a generic claim, provided that the effect is sufficiently demonstrated to characterize a generic invention." Also, the scope of enablement must only bear a "reasonable correlation" to the scope of the claims. *See, e.g., In re Fisher*, 427 F.2d 833, 166 (CCPA 1970).

Furthermore, Lee et al., *Biology of Reproduction* 72:42-49 (2005) have shown that human uterine endometrial cells can be used as feeders for the establishment and culture of human embryonic stem cell lines. Other establishment methods are also described in Ellerstrom et al., *Stem Cells* 24:2170-2176 (2006). Copies of the Lee et al. and Ellerstrom et al. papers are submitted herewith for the Examiner's reference.

Thus, the methods recited in the present claims are enabled commensurate in scope with the claims. Accordingly, Applicant respectfully requests reconsideration and withdrawal of this rejection.

## VI. Response to Claim Rejections Under 35 U.S.C. § 112, Second Paragraph

Claims 1-21, 35, and 57-61 have been rejected under 35 U.S.C. § 112, second paragraph, as purportedly indefinite for the following reasons:

*A.* The Examiner has stated that claims 1-4 are confusing because although the preamble recites that the stem cell line is human, the method steps themselves are not directed to a human stem cell line.

In response, claims 1-4 have been amended to recite that the oocytes, blastocyts, and resultant stem cell lines are human, as suggested by the Examiner.

***B.*** The Examiner has indicated that the phrase "less than about" renders claims 9, 60, and 61 indefinite, because the phrase is relative.

In response, claims 4, 9, 60, and 61 have been amended by deleting the word "about."

***C.*** The Examiner has stated that the limitation "the stem cells" in claims 9 and 10 lacks antecedent basis.

In response, claims 9 and 10 have been amended by replacing "the stem cells" with "the pluripotent human blastocyst-derived stem cell line."

***D.*** The Examiner has stated that claims 11 and 16 are confusing because it is not clear which "feeder cells" are referred to.

In response, claim 11 has been amended by clarifying that the feeder cells referred to in the claim are the feeder cells in step iv). In addition, claim 16 has been amended to recite that the feeder cells of at least one of step ii) and step iv) are embryonic feeder cells.

***E.*** The Examiner has stated that claim 14 is unclear because it recites the term "and/or."

In response, claim 14 has been amended by replacing the term "and/or" with the phrase "at least one of."

***F.*** The Examiner has stated that claim 18 is unclear because it is not clear whether the claim is intended to limit the feeder cells of step ii) or step iv).

In response, claim 18 has been amended to recite that "the feeder cells of at least one of step ii) and step iv) are of mouse or human origin."

In view of the above, Applicant respectfully requests reconsideration and withdrawal of the objections to the claims.

## VII. Response to Claim Rejection Under 35 U.S.C. § 102

Claim 21 has been rejected under 35 U.S.C. § 102(b) as allegedly being anticipated by "Thomson (1998)" (Thomson et al., Science 282, 1145-1147 (1998)).

To expedite prosecution in the present application, and not to acquiesce to the Examiner's rejection, claim 21 has been canceled. Thus, this rejection has been rendered moot.

**VIII. Response to Claim Rejections Under 35 U.S.C. § 103**

*A.* Claims 1-3, 5-7, 12, 13, 16-20, 21 and 57-59 have been rejected under 35 U.S.C. § 103(a) as allegedly being unpatentable over "Thomson (2001)" (U.S. Patent No. 6,200,806, issued March 13, 2001) when taken with Thomson (1998), Rijnders et al. (Human Reproduction 13, 2869-2873 (1998)), and Lanzendorf et al. (Fertility and Sterility 76, 132-137 (July 2001)), as evidenced by Stem Cell Information (National Institutes of Health, Appendix C, pages 1-4, accessed online at http://stemcells.nih.gov/info/scireport/appendixC.asp, January 31, 2007). This rejection is respectfully traversed, for at least the following reasons:

According to the Examiner, Thomson (2001) teaches methods for the production of primate embryonic stem cells, (ES cells), including human ES cells. However, Thomson (2001) only exemplifies the preparation of embryonic stem cell lines from the Rhesus Macaque Monkey (col. 14, lines 12-1 5). Thomson (2001) states that the methods shown for the Rhesus Macaque Monkey can be used to derive human ES cell lines (*see* col. 7 lines 4-10). However, the reference does not provide any evidence. Applicant submits that because the establishment of pluripotent blastocyst-derived stem cell lines is an extremely delicate and species-specific procedure, a person of ordinary skill in the art would not have reasonably predicted that a method established for isolating and maintaining a culture of pluripotent blastocyst-derived stem cells for one species would succeed when transferred to a different species.

WO 99 27076 (cited in the International Search Report dated July 22, 2003), for example, indicates that it is not straightforward to extrapolate the procedure for establishing ES cell lines in mice to a procedure for establishing ES cell lines in rats, despite the fact that mice and rats are evolutionary closely related (*see* page 1, line 27, to page 2, line 3 and page 2, line 29, to page 3 line 4). Accordingly, a method for obtaining a pluripotent human blastocyst-derived stem cell line would not be obvious from the teaching of the preparation of ES cells from Rhesus Macaque Monkey.

Thomson (2001) further supports the position that methodologies for the establishment of ES cells in one species are not readily transferable to other species. In particular, the reference teaches that while methods for obtaining murine blastocyst-derived stem cell lines had been available at least as early as 1981 (*see* col. 1 lines 34-38), a pluripotent stem cell line derived from embryos of another species (hamster) was not derived until 1988 (col., 3 lines 61-65). Similarly, US 5,905,042 (also cited in the International Search Report) discloses that methods for deriving embryonic stem cells *in vitro* from early preimplant mouse embryos had been well known since 1981, but the earliest date for the isolation of (purportedly) pluripotent embryonic stem cell lines in another mammalian species (pigs) is 1990 (col. 1, line 67 - col. 2, line 3). Thus, it is evident that the methodologies established in 1981 for isolating and culturing murine blastocyst-derived pluripotent stem cell lines were not easily transferable from one species to another; otherwise, it could reasonably be assumed, cultured blastocyst-derived stem cell lines from species other than mouse would have been made available more quickly than was actually the case, particularly in light of the scientific importance of work in this area.

In addition, the isolation of ICM cells according to the present invention is performed by mechanical dissection, without the use of enzymes and/or antibodies (*see, e.g.,* claim 1 and page 5, lines 7-11 of the specification). In contrast to the present invention, Thomson (2001) fails to teach or suggest the isolation of inner cell mass (ICM) cells by manual, or mechanical, dissection. Instead, the reference clearly discloses that ICM-derived masses are removed from endoderm outgrowth with a micropipette and then exposed to 0.05% trypsin-EDTA supplemented with 1% chicken serum for 3-5 minutes. Given the above-mentioned delicacy and unpredictability of methods for obtaining pluripotent stem cell lines from blastocysts, it would not have been obvious to one of ordinary skill in the art to modify the method of Thomson (2001) by manually or mechanically dissecting the ICM-derived mass instead of dissociating them using Trypsin-EDTA supplemented with chicken serum.

The Examiner has further relied on Rinjders and Lazendorf. However, these references do not remedy the serious deficiencies of Thomson (2001) and Thomson (1998). The article by Rinjders concerns the investigation of the predictive value of embryo morphology at a relative early stage for blastocyst formation and subsequent ability to implant in patients (page 2871, right column, lines 5-7). Lazendorf teaches the selection of

Grade I and 2 blastocysts followed by spontaneous hatching or removal of the zona pellucida and treatment with acidified Tyrode's medium. In both references, immunosurgery was used to isolate the ICM cells, and the references do not teach or suggest mechanical dissection. In addition, neither of the articles suggests combining the use of oocytes of grade 1 or 2 to obtain a blastocyst of grade A or B in the method of Thomson (2001).

In particular, the present specification discloses that mechanical dissection may be performed using a knife or glass capillary. In addition, other methods for mechanical dissection will be apparent to those skilled in the art. What is important to notice is that the colonies are mechanically dissected into pieces, and not "pipetted" or otherwise dissociated or disaggregated into separate individual cells. Thus, the present invention represents a significant step forward towards a procedure that is free of "xeno" material, as compared to the prior art that involves the use of non-human immunosurgical materials. Further, the manual dissection of cell colonies is also practical in the sense that it can readily be performed by those skilled in the art to produce large numbers of cells.

Applicant also notes that the Examiner has stated that the cell line described in Thomson (1998) has a proliferation capacity of more than 21 months as evidenced by NIH Stem Cell Information. However, there is no evidence that this "H9" cell line is stable after proliferation for more than the approximately 8 months (32 passages) stated in Thomson (1998) at page 1145, col. 3, lines 1-5. In particular, no karyotypes, stem cell specific immuno markers nor pluripotency *in vitro* or *in vivo* have been shown. Karyotypic aberrations are commonly observed when stem cells have been passaged enzymatically or chemically (*see, e.g.,* page 20, column 2, lines 3-14 of Mitalpova et.al., *Nature Biotechnology* 23:19-20 (2005) and page 136, lines 1-17 of Caisander et al., *Chromosome Research* 14:131-137 (2006) (copies of which are submitted herewith), and in view of this it is likely that the H9 cell line carries an abnormal phenotype (*i.e.* the H9 cell line is not "stable").

In contrast to the procedure taught in Thomson (1998), the present method enables the establishment of large numbers of stable pluripotent human embryonic stem cell lines. The issue of "stability" is discussed in some detail in the application, from which it will be clear to the skilled reader that what is intended is a pluripotent human embryonic stem cell line that remains undifferentiated and exhibits no chromosomal instabilities for a period of more than twenty-one months. This is especially advantageous, because it enables the generation of substantial numbers of cells which may be produced in "lots," each lot comprising a number

of individual units (*e.g.* plates, straws, or the like) of similar cells. The generation of large numbers of stable, undifferentiated cells is attractive from a commercial point of view, and the ability to produce such large numbers of cells in lots is especially advantageous because an individual unit may be withdrawn from each lot for characterization of the remaining units within the lot. Such characterization of the undifferentiated embryonic stem cells is particularly desirable from a scientific perspective, allowing the end-user of the cells to know much more about the cells.

By using the method of the present invention, Applicant was able to produce some 2,473 culture units in 2004 and some 3,833 units in 2005, including five lots of units in 2005 and three lots in 2006 to date, each lot comprising 100 frozen straws. The Examiner will appreciate that this is a very significant achievement, and indeed Applicant is unaware of any other entity in the world that is presently able to produce such large quantities of undifferentiated human embryonic stem cells free from chromosomal instabilities.

In view of the above, Applicant submits that the subject matter recited in the present claims is not obvious over the references cited by the Examiner. Accordingly, Applicant respectfully requests reconsideration and withdrawal of this rejection.

***B.*** Claims 4, 8-10, 60 and 61 have been rejected under 35 U.S.C. § 103(a) as allegedly being unpatentable over Thomson (2001) when taken with Thomson (1998), Rijnders et al. (Human Reproduction 13, 2869-2873 (1998)), Lanzendorf et al. (Fertility and Sterility 76, 132-137 (July 2001)), and Marshall et al. (Methods in Molecular Biology 158, 11-18 (January 2001)). This rejection is respectfully traversed, for at least the following reasons:

As discussed above, it would not have been obvious for one with ordinary skill in the art to combine the teaching of Thomson (2001), which involves preparation of embryonic stem cell lines from the Rhesus Macaque Monkey, with the teaching of Rinjders, concerning the predictive value of human embryo morphology for blastocyst formation and subsequent implant in patients, and Lazendorf, concerning selection of Grade 1 and 2 human blastocysts followed by spontaneous hatching or removal of the zona pellucida, and Thomson (1998), concerning the proliferative capacity, in order to obtain the present invention. Marshall et al. does not remedy the serious deficiencies of Thomson (2001), Thomson (1998), Rijinders et al., and Lanzendorf et al.

Furthermore, similar to Applicant's position with regard to Thomson (2001), the teaching in Marshall with regard to the isolation and maintenance of Rhesus Macaque Monkey cells is not readily transferable to a human blastocyst-derived stem cell line. Additionally, claim 4 recites a method including selecting the starting material very carefully in order to enable a suitable result (*i.e.* only specific qualities of oocytes and/or blastocysts may be used), and using specific conditions during the propagations of the cells in order to enable a suitable result (*i.e.* specific densities of the feeder cells). The skilled person would not take the above-referenced documents, alone or in combination, and be motivated to arrive at the specific conditions recited in claim 4.

Accordingly, Applicant submits that the person skilled in the art would not have predicted that the methods of claims 4, 8-10, 60 and 61 would be transferable to a human blastocyst-derived sterm cell line. Thus, Applicant respectfully requests reconsideration and withdrawal of this rejection.

*C.* Claim 11 has been rejected under 35 U.S.C. § 103(a) as allegedly being unpatentable over Thomson (2001) when taken with Thomson (1998), Rijnders et al., Lanzendorf et al., and Conner (Current Protocols in Molecular Biology, 23.2.1-23.2.7 (2000)). This rejection is respectfully traversed for at least the following reasons:

As discussed above, it would not be obvious for one with ordinary skill in the art to combine the teaching of Thomson (2001), which involves preparation of embryonic stem cell lines from the Rhesus Macaque Monkey, with the teaching of Rinjders, concerning the predictive value of human embryo morphology for hlastocyst formation and subsequent implant in patients, and Lazendorf, concerning selection of Grade 1 and 2 human blastocysts followed by spontaneous hatching or removal of the zona pellucida, and Thomson (1998), concerning the proliferative capacity, in order to obtain the present invention. Conner does not remedy the serious deficiencies of Thomson (2001), Thomson (1998), Rijinders et al., and Lanzendorf et al.

Furthermore, although Conner may provide guidance on the use of MEF cells and suggest that early passage of cells must be used for ES cell growth, the reference does not show that this is the case for human blastocyst-derived stem cell lines (*see* page 23). Given at

least the fact that methodologies are not readily transferable from one species to another, it is Applicant's position that claim 11 would not have been obvious to a person skilled in the art.

Thus, Applicant respectfully requests reconsideration and withdrawal of this rejection.

***D.*** Claims 14 and 15 have been rejected under 35 U.S.C. § 103(a) as allegedly being unpatentable over Thomson (2001) when taken with Thomson (1998), Rijnders et al., Lanzendorf et al., Gardner et al. (Human Reproduction, 13, 148-60 (1998)), and Gardner et al. (Human Reproduction, 14, 2575-2580 (1999)). This rejection is respectfully traversed, for at least the following reasons:

As noted above, it would not be obvious for one with ordinary skill in the art to combine the teaching of Thomson (2001), which involves preparation of embryonic stem cell lines from the Rhesus Macaquc Monkey, with the teaching of Rinjders, concerning the predictive value of human embryo morphology for blastocyst formation and subsequent implant in patients, and Lazendorf concerning selection of Grade 1 and 2 human blastocysts followed by spontaneous hatching or removal of the zona pellucida, and Thomson (1998), concerning the proliferative capacity, in order to obtain the present invention. The Gardner et al. references do not remedy the serious deficiencies of Thomson (2001), Thomson (1998), Rijinders et al., and Lanzendorf et al.

Accordingly, Applicant respectfully requests reconsideration and withdrawal of this rejection.

***E.*** Claim 35 has been rejected under 35 U.S.C. § 103(a) as allegedly being unpatentable over Thomson (2001) when taken with the Stratagene Catalog (page 39, (1998)). This rejection is respectfully traversed, for at least the following reasons:

Even though the Stragtagene Catalog describes the principle behind a kit, it is Applicant's position that the teaching of Thomson (2001) is not readily transferable to a stem cell line from another species.

Thus, Applicant respectfully requests reconsideration and withdrawal of this rejection.

## CONCLUSION

In view of the foregoing, further and favorable action in the form of a Notice of Allowance is believed to be next in order. Such action is earnestly solicited.

In the event that there are any questions relating to this Amendment and Reply or the application in general, it would be appreciated if the Examiner would telephone the undersigned attorney so that prosecution of this application may be expedited.

Respectfully submitted,

BUCHANAN INGERSOLL & ROONEY PC

Date: July 12, 2007

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# Derivation of a Xeno-Free Human Embryonic Stem Cell Line

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# Derivation of a Xeno-Free Human Embryonic Stem Cell Line

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Key Words. Human embryonic stem cell • Human serum • Human feeders • Clinical therapies

ABSTRACT

**Elimination of all animal material during both the derivation and long-term culture of human embryonic stem cells (hESCs) is necessary prior to future application of hESCs in clinical cell therapy. The potential consequences of transplanting xeno-contaminated hESCs into patients, such as an increased risk of graft rejection [STEM CELLS 2006;24:221–229] and the potential transfer of nonhuman pathogens, make existing hESC lines unsuitable for clinical applications. To avoid xeno-contamination during derivation and culture of hESCs, we first developed a xeno-free medium supplemented with human serum, which supports long-term (>50 passages) culture of hESCs in an undifferentiated state. To enable derivation of new xeno-free hESCs, we also established xeno-free human foreskin fibroblast feeders and replaced immunosurgery, which involves the use of guinea pig complement, with a modified animal-product-free derivation procedure. Here, we report the establishment and characterization (>20 passages) of a xeno-free pluripotent diploid normal hESC line, SA611.** STEM CELLS *2006;24:2170–2176*

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## INTRODUCTION

Human embryonic stem cells (hESCs) hold great promise for future clinical cell therapies in the fields of, for example, diabetes, cardiac infarction, and neurodegenerative diseases, because of their unique potential to differentiate into all cell types found in the human body. In addition, it has recently been revealed that hESCs and their differentiated derivatives are less susceptible to immune rejection than adult cells [1], which is encouraging news for future clinical application. However, the majority of hESC lines available to date have been directly or indirectly exposed to animal material during their derivation and/or propagation in vitro. Transplanting xeno-contaminated hESCs to patients will increase the risk of graft rejection [2] and transfer of nonhuman pathogens, suggesting that the existing hESC lines are unsuitable for clinical applications. Closer inspection of previous attempts to establish xeno-free hESC lines reveals, in each case, that not all animal products had been replaced. For example, several recent hESC derivation reports [3–5] used Knock-Out serum replacement (SR), which contains animal protein and is a source of nonhuman sialic acid [2]. Although Richards et al. [6] used human serum (HS) and human feeder cells, and Ludwig et al. [7] recently presented the derivation of two human ES cell lines (WA15 and WA16) in a chemically defined cell culture system, they both used immunosurgery to isolate the inner cell mass (ICM). Immunosurgery is a method commonly used to remove the outer trophectoderm epithelial cell layer from the blastocyst by incubation in polyclonal rabbit anti-human whole-serum antibodies and guinea pig complement. Consequently, although these culture systems may be free of animal products, the derivation procedure is not, and therefore, these hESC lines should be considered potentially xeno-contaminated.

To obtain a truly xeno-free system for the derivation and maintenance of hESCs, three major sources of xeno-contamination must be eliminated. First, stable long-term maintenance of self-renewing and pluripotent hESCs traditionally involves the use of feeder cells. Most commonly used feeder cells are either of animal origin, such as mouse embryonic fibroblasts (MEFs), or have been exposed to animal proteins (e.g., by the use of fetal bovine serum [FBS]) during their isolation and culture procedure. Second, the medium supplements necessary for the cultivation of hESCs, such as FBS or SR, contain various animal proteins. Third, trophectoderm removal to isolate the ICM is traditionally performed by immunosurgery as described above. Several groups have attempted to exclude individual animal components by using feeder-free matrices [3, 7, 8], feeder cells of human origin [6, 9–12], or defined xeno-free media [7]. However, to date, it has not been possible to fully eliminate all animal material during both derivation and culturing of hESCs in order to create a completely xeno free system.

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To completely avoid exposure of hESCs to animal products, we developed a xeno-free protocol for the derivation and culture of new hESC lines. All animal-derived and animal-exposed components have been meticulously replaced by tested human-derived or recombinant/synthetic substances. Initially, a xeno-free culture environment based on a human feeder layer in combination with a HS-supplemented medium was designed and tested extensively. The culture system proved suitable to support the stable maintenance of self-renewal and pluripotency of previously established hESCs for more than 50 passages. To replace the xeno-contaminated commercially available human feeder cells, we then established new primary cultures of human foreskin fibroblast (HFF) feeders under xeno-free conditions. Finally, to derive new xeno-free hESCs, immunosurgery was replaced with a xeno-free alternative method.

We now report the successful derivation of the first xeno-free hESC line, which has been established and cultured under truly animal protein-free conditions. To date, the xeno-free hESC line SA611 has been maintained in culture for over 30 consecutive passages. Characterization of SA611 shows that the cell line is genetically normal and exhibits the characteristics of undifferentiated pluripotent hESCs.

## Materials and Methods

### Human Material

Human blood from healthy donors and surplus human embryos from clinical in vitro fertilization (IVF) treatment were donated after informed consent and approval of the local ethics committee at Göteborg University.

### Preparation of HS

HS was prepared as previously described [13]. For the preparation of each batch of serum, human blood was collected from 15 healthy blood donors at the hospital's blood center. All donors were from the general public and belonged to the regular registered blood donors at the hospital. The blood was collected in a transfusion bag (Dry Pack: JMS, Singapore, http://www.jmss.com) blended, and stored overnight at 4–8°C before centrifugation at 1,000*g* for 8 minutes. Serum was pooled, sterile-filtered, and frozen until use. Superior quality human serum was repeatedly produced in our laboratory from donated human blood, which was tested for standard pathogens (hepatitis B and C, HIV, human T-cell leukemia virus type 1, and syphilis) at the hospital's blood center.

### Culture of Commercial Non-Xeno-Free HFF Feeders

Commercially available non-xeno-free HFFs were obtained from the American Type Culture Collection (CRL-2429; American Type Culture Collection, Manassas, VA, http://www.atcc.org). After expansion, confluent monolayers of HFFs were treated with mitomycin (Mutamycin; Bristol-Myers Squibb, Princeton, NJ http://www.bms.com)-C (Sigma-Aldrich, Stockholm, Sweden, http://www.sigmaaldrich.com) and plated on 0.1% gelatin (Sigma-Aldrich)-coated IVF wells (200,000 cells per 2.89 cm$^2$) in the xeno-free medium based on knockout-Dulbecco's modified Eagle's medium (KO-DMEM) (Gibco, Paisley, Scotland, http://www.invitrogen.com) supplemented with 20% HS, 4 ng/ml human recombinant (hrb) fibroblast growth factor (FGF) (Invitrogen, Carlsbad, CA, http://www.invitrogen.com), 1% penicillin-streptomycin, 1% Glutamax, 0.5 mmol/l β-mercaptoethanol, and 1% nonessential amino acids (all reagents from Invitrogen).

### Culture of hESCs on Commercial HFFs in HS-Supplemented Medium

The hESC line SA121 [14] (Cellartis, Göteborg, Sweden, http://www.cellartis.com), which originally was established and maintained on MEF feeders in VitroHES medium (Vitrolife AB, Kungsbacka, Sweden, http://www.vitrolife.com), was transferred onto mitotically inactivated commercial HFFs (CRL-2429) in KO-DMEM (Invitrogen) supplemented with 10% HS, 4 ng/ml hrbFGF (Invitrogen), 1% penicillin-streptomycin, 1% Glutamax, 0.5 mmol/l β-mercaptoethanol and 1% nonessential amino acids (all reagents from Invitrogen). The hESCs were mechanically passaged to new culture dishes with fresh feeders every 5–7 days using a Stem Cell Tool (Swemed Lab International AB, Billdal, Sweden, http://www.swemed.com).

### Derivation and Culture of Xeno-Free HFF Feeders

Under Swedish legislation, the use of nontraceable human surplus material that would otherwise be discarded does not require human subjects approval from the ethics committee. Human foreskin from circumcised infant boys was aseptically collected in Iscove's modified Dulbecco's medium (IMDM) (Invitrogen) + 1% penicillin-streptomycin. The skin explants were cut into small pieces using a sterile scalpel and placed into 25-cm$^2$ tissue culture flasks containing 4 ml of IMDM with 1% penicillin-streptomycin (Invitrogen) and 10% HS. After approximately 10 days, a confluent monolayer of primary HFFs was established. For subsequent expansion, the HFF cells were dissociated to single cells using a recombinant animal protein-free enzyme, TrypLE Select (Invitrogen) and passaged into 75-cm$^2$ flasks. Confluent monolayers of HFFs were treated with mitomycin-C (Sigma-Aldrich) and plated on 0.1% hrb gelatin (FibroGen, CA, http://www.fibrogen.com)-coated IVF wells (200,000 cells per 2.89 cm$^2$).

### Establishment and Culture of Xeno-Free hESCs

Donated embryos were cultured to the blastocyst stage as described previously [14]. To eliminate the zona pellucida and to damage the trophectoderm, blastocysts were incubated in acid Tyrode's solution (Medicult, Mollehaven, Denmark, http://www.medicult.com). Subsequently, zona-removed blastocysts were placed onto inactivated xeno-free HFFs. The culture medium consisted of KO-DMEM (Invitrogen) supplemented with 20% HS, 10 ng/ml hrbFGF (Invitrogen), 1% penicillin-streptomycin, 1% Glutamax, 0.5 mmol/l β-mercaptoethanol, and 1% nonessential amino acids (all reagents from Invitrogen). Fifty percent of the medium was renewed every 2–3 days. After 10 days, the cells were mechanically passaged to fresh feeders. At passage 2, the hESCs (SA611) were enzymatically dissociated once using TrypLE Select in order to release the growing hESCs from an overgrowth of differentiated cells. Earlier attempts to perform this release by mechanical manipulation had been unsuccessful. From passage 3 on, SA611 was mechanically passaged approxi



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Table 1. Long-term validation of morphology and marker expression in human embryonic stem cell line SA121 cultured on human foreskin fibroblast and in human serum-supplemented medium[a]

| Passage | Morphology | Oct-4 | Tra-1-60 | Tra-1-81 | SSEA-1 | SSEA-3 | SSEA-4 | ALP |
|---|---|---|---|---|---|---|---|---|
| 5 | Undifferentiated | + | + | + | | + | + | + |
| 12 | Undifferentiated | + | + | + | − | + | + | + |
| 22 | Undifferentiated | + | + | + | − | + | + | + |
| 41 | Undifferentiated | + | + | ND | ND | ND | ND | + |
| 50 | Undifferentiated | + | + | + | − | + | + | ND |

[a]+, >90% of the cells are positive; −, <5% of the cells are positive.
Abbreviation: ND, not determined.

mately once a week. To eliminate the risk of cross-contamination the xeno-free HFFs, donated blastocysts, and the derived xeno-free hESC line SA611 were cultured and handled physically separated from all non-xeno-free cultures and components. Eleven fresh blastocysts that could not be used in infertility treatment were treated and cultured under identical conditions.

### Immunohistochemical and Histochemical Analysis of hESCs

hESC cultures were fixed in 4% paraformaldehyde for 15 minutes, permeabilized for 5 minutes in 0.5% Triton X-100 solution (Sigma-Aldrich), and blocked in 5% FBS in phosphate-buffered saline (Invitrogen). The cells were incubated with primary antibodies (Oct-4, TRA-1-60, TRA-1-81, SSEA-1, SSEA-3, and SSEA-4; Santa Cruz Biotechnology, Santa Cruz, CA, http://www.southernbiotech.com) overnight at 4°C, and visualized by incubation in fluorescein isothiocyanate (FITC)- or Cy3-conjugated secondary antibodies (Jackson Immunoresearch Laboratories, West Grove, PA, http://www.jacksonimmuno.com) for 60 minutes at room temperature. Cell nuclei were counterstained with 4,6-diamidino-2-phenylindole (Sigma-Aldrich). The activity of alkaline phosphatase was determined using an alkaline phosphatase activity detection kit according to the manufacturer's instructions (Sigma-Aldrich). Stainings were evaluated and documented using a Nikon Eclipse TE-2000 U fluorescence microscope (Nikon, Tokyo, http://www.nikon.com).

### Genetic Characterization

For karyotype analysis, the hESCs were incubated in the presence of colcemid, trypsinized, fixed, and mounted on glass slides. The chromosomes were visualized by DAPI staining, arranged, and documented using an inverted microscope equipped with appropriate filters and software (CytoVision; Applied Imaging, Santa Clara, CA, http://www.appliedimagingcorp.com). SA121 was analyzed after passage 30 (eight metaphases screened) and at passage 50 (19 metaphases screened). SA611 was analyzed at passage 9 (15 metaphases screened) and at passage 22 (13 metaphases screened).

For fluorescence in situ hybridization (FISH) analysis, the commercially available preimplantation genetic testing (includes chromosomes 13, 18, 21, and X and Y) multiprobe panel and chromosome enumerating probe (for chromosomes 12, 17, and 20) kits (Vysis, Downers Grove, IL, http://www.vysis.com) containing probes for chromosomes 12, 13, 17, 18, 21, X, and Y

Table 2. Long-term validation of chromosomal stability and pluripotency in human embryonic stem cell line SA121 cultured on human foreskin fibroblast and in human serum-supplemented medium

| Passage | Karyotype | FISH | Teratoma |
|---|---|---|---|
| 0[a] | 46 XY | 2n XY[b] | Endo/Ecto/Meso |
| >30 | 46 XY | 2n XY[c] | Endo/Ecto/Meso |
| 50 | 46 XY | 2n XY[d] | ND |

[a]Heins et al. [14].
[b]Chromosomes 13, 18, 21, X, and Y were analyzed.
[c]Chromosomes 12, 13, 18, 20, 21, X, and Y were analyzed.
[d]Chromosomes 12, 13, 17, 18, 20, 21, X, and Y were analyzed.
Abbreviations: Ecto, ectoderm; Endo, endoderm; Meso, mesoderm; ND, not determined.

were used with minor modifications. The slides were analyzed in an inverted microscope equipped with appropriate filters and software (CytoVision). For each probe, a minimum of 100 nuclei were analyzed. In most cases, 200 nuclei were analyzed. Analysis of SA121 was performed after 30 and 50 passages. SA611 was analyzed at passages 9 and 22.

### Analysis of Pluripotency In Vitro

Pluripotency was tested in vitro by spontaneous differentiation of hESCs on the feeder layer or after transfer to Matrigel plates. After 2–4 weeks, fixed and differentiated colonies were analyzed immunohistochemically to identify cells derived from the three germ layers. The following primary antibodies were used: Foxa2 (Santa Cruz Biotechnology), $\alpha$ smooth-muscle actin (Chemicon, Temecula, CA, http://www.chemicon.com), and $\beta$-tubulin III (Sigma-Aldrich). Alexa (Molecular Probes Inc., Eugene, OR, http://probes.invitrogen.com) and FITC-conjugated secondary antibodies (Jackson Immunoresearch Laboratories) were used for detection. Fixation and incubation was performed as described above.

### Analysis of Pluripotency In Vivo

Pluripotency in vivo was assessed by teratoma formation in severe combined immunodeficient (SCID) mice as described earlier [14]. In brief, undifferentiated hESC colonies were mechanically cut into 200-$\mu$m × 200-$\mu$m pieces and surgically placed under the kidney capsule of SCID mice (C.B-17/IcrCrl-scidBR; Charles River Laboratories, Sulzfeld, Germany, http://www.criver.com). The mice were sacrificed after 8 weeks, and tumors were excised and fixed in 4% paraformaldehyde. Hematoxylin and eosin-stained paraffin sections were evaluated his-

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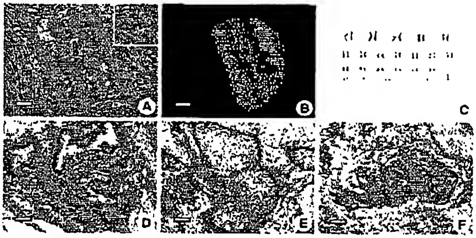

Figure 1. Long-term validation of a culture system for human embryonic stem cells (hESCs) (SA121) based on human foreskin fibroblast and human serum. (A): Morphology of hESC line SA121 after 40 passages. Inset, ×2 detail magnification. (B): Immunofluorescence staining of undifferentiated SA121 cells after >40 passages with anti-Oct4 antibodies. (C): Diploid normal karyotype of SA121 after >50 passages. (D–F): Histological analysis of teratomas derived from SA121 after 33 passages (D). Secretory epithelium (endoderm). (E): Cartilage (mesoderm). (F): Neuroectoderm (ectoderm). Scale bars = 100 μm (A, B), 50 μm (D–F).

tologically for the presence of differentiated human tissue derived from all three embryonic germ layers, such as neuroectoderm, cartilage, and gut-like epithelium.

## Results

### Validation of a hESC Culture System Based on HFF Feeders and HS-Supplemented Medium

To culture hESCs in the absence of animal cells and protein, we used a culture system based on HFF feeders and HS-supplemented culture medium. To validate the stability of such culture environment, colonies from hESC line SA121 [14] were transferred from the traditional culture system (MEF feeders in VitroHES medium) [14] to the new culture system, to test its ability to support maintenance of undifferentiated pluripotent hESCs for more than 50 consecutive passages. Based on the morphological appearance of the colonies, the hESCs required 3–5 passages to adjust to the new environment after the initial transfer. At regular intervals throughout the validation study (>50 passages), the cultures were evaluated morphologically, genetically, by marker expression analysis, and by teratoma formation. The results are summarized in Tables 1 and 2. At all passages analyzed, the cells expressed the expected stem cell markers Oct-4 (Fig. 1B; Table 1), TRA1-60, TRA1-81, SSEA-3, and SSEA-4 (Table 1), whereas they were negative for SSEA-1 (Table 1), a marker of differentiated hESCs. In addition, the colonies showed positive staining for alkaline phosphate activity (Table 1). At all passages analyzed throughout the validation study, hESCs maintained a stable diploid normal karyotype (46 XY) (Fig. 1C; Table 2). FISH analysis on selected chromosomes confirmed this finding (Table 2). Pluripotency was tested after 33 passages, demonstrating that the new culture environment supported growth of pluripotent hESCs; that is, histochemical analysis of teratomas revealed the presence of derivatives of endoderm (gut-like epithelium; Fig. 1D), mesoderm (cartilage; Fig. 1E), and ectoderm (neuroectoderm; Fig. 1F, Table 2).

### Derivation of Xeno-Free HFFs

After successful validation of the culture system we proceeded to replace the commercially available human feeders with xeno-free HFFs. HFF primary cultures were established from material obtained from circumcisions. Establishment and culture of HFFs was performed under strictly xeno-free conditions in culture medium supplemented with 10% HS. During the first passages, individual cells with epitheloid morphology were visible among the fibroblasts, but from passage 3 on, all the cells in culture revealed homogenous fibroblastic morphology. For the xeno-free derivation and culture of hESCs, we only used xeno-free feeders from passages 5–8 that had been batch tested for their ability to support undifferentiated growth of hESCs. It has been reported earlier that HFFs can be expanded in vitro for at least 42 passages [10, 11] before senescence, but since each donated skin sample gave rise to abundant numbers of primary HFFs, we did not feel the need to evaluate higher passage feeders.

### Development of a Xeno-Free Alternative for the Isolation of the ICM

To prevent xeno-contamination during the establishment procedure, we replaced the commonly used combination of pronase digestion of the zona pellucida and immunosurgical removal of the trophectoderm by an approach that uses acid Tyrode's solution to dissolve the zona pellucida and to damage the trophectoderm. To avoid damage to the ICM while achieving complete removal of the zona pellucida, as well as partial destruction of the underlying trophectodermal cell layer, in a single step, exposure to the acidic solution (pH of 2.5–3) was carefully optimized by incubation for increasing periods of time, while the morphology of the zona pellucida, trophectoderm, and ICM were monitored by visual microscopical inspection. The optimal incubation time was found to be in the range of 30–45 seconds.



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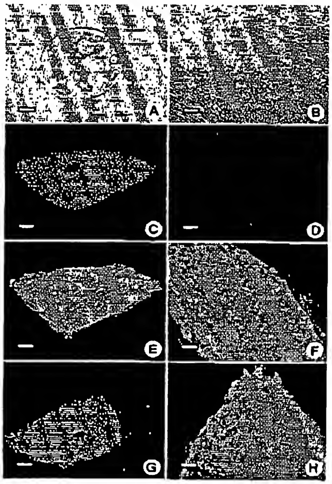


**Figure 2.** Morphological and immunohistochemical characterization of the xeno-free human embryonic stem cell (hESC) line SA611. **(A):** The blastocyst of SA611 (scale bar = 25 $\mu$m). **(B):** Morphology of hESC line SA611 after 12 passages under xeno-free conditions (scale bar = 100 $\mu$m). **(C–H):** Immunofluorescence stainings of undifferentiated SA611 cells after 12 passages using Oct-4 (C), SSEA-1 (D), TRA1-60 (E), TRA1-81 (F), SSEA-3 (G), and SSEA-4 (H) antibodies. Note that images in (C) and (E) are images of a double staining. Scale bars = 50 $\mu$m (C–E, G). 100 $\mu$m (F, H).

### Establishment, Expansion, and Cryopreservation of a Xeno-Free hESC Line

To derive a new hESC line under xeno-free conditions, donated fresh blastocysts (Fig. 2A) were treated with acid Tyrode's solution under visual inspection and placed onto xeno-free HFFs. Initially, outgrowth of hESC-like cells from the ICM was accompanied by the appearance of differentiated cells, presumably representing primitive endoderm and trophectoderm-derived cell types. During subsequent mechanical passaging of hESC-like cells, the differentiated cells disappeared. The new hESC cell line SA611 forms distinct colonies with clearly defined borders, and the cells exhibit the characteristic morphology of hESCs, that is, densely packed cells with a high nucleus-to-cytoplasm ratio (Fig. 2B). To date, line SA611 has been in culture for over 8 months and has been propagated for more than 30 passages under xeno-free culture conditions. At passages 6, 7, 20, and 25, SA611 was cryopreserved in completely sealed straws by vitrification. Validation of the process by subsequent thawing of individual straws resulted in efficient recovery of viable, undifferentiated hESCs.

### Characterization of the Xeno-Free hESC Line SA611

To confirm the undifferentiated state of SA611, the cultures were evaluated by immunofluorescence at passages 5, 12, and 22. SA611 colonies uniformly expressed the expected markers of undifferentiated hESCs, including Oct-4, TRA1-60, TRA1-81, SSEA-3, and SSEA-4, whereas they were negative for SSEA-1 (Fig. 2C–2H). The hESC colonies also displayed strong alkaline phosphatase activity (data not shown).

Karyotype analyses at passages 9 and 22 showed that SA611 maintained a stable diploid normal karyotype (46 XY) (Fig. 3A). FISH analyses at passages 9 and 22 on selected chromosomes (12, 13, 17, 18, 21, X, and Y) confirmed this finding (Fig. 3B, 3C).

The pluripotency of line SA611 was evaluated in vitro as well as in vivo. Initially, spontaneous differentiation of hESCs in vitro was analyzed in high-density cultures on HFFs as well as after transfer of colonies to Matrigel. The appearance of $\beta$-III-tubulin$^+$ neurons, ASMA$^+$ smooth muscle cells, and Foxa2$^+$ cells indicated that SA611 were able to differentiate into derivatives of all three embryonic germ layers in vitro (Fig. 4B, 4D, 4F). To explore the pluripotent nature of SA611 in vivo, clusters of undifferentiated hESCs were grafted under the kidney capsule of SCID mice. The appearance of ectodermal (neuroectoderm; Fig. 4A), mesodermal (cartilage; Fig. 4C), and endodermal (gut-like epithelium; Fig. 4E) tissues within the teratomas demonstrated that SA611 exhibits the characteristic in vivo differentiation capacity of pluripotent hESCs.

In summary, the xeno-free hESC line SA611 stably expressed the genetic and phenotypic characteristics of undifferentiated pluripotent human stem cells.

## Discussion

To date, all reported hESC lines have been exposed to animal material at some point during their derivation [e.g., 6, 7] or cultivation [e.g., 2]. To develop a reproducible xeno-free procedure for deriving and culturing hESCs, it is necessary to first show that all steps in the derivation, passaging, and culturing of hESCs are completely free of animal products. The second issue, which relates to the stability of the derivation and culture system, is to maintain the cells under the tested defined conditions long enough to ensure that the system is phenotypically and genetically stable.

The major sources of xeno-contamination are the presence of animal feeders and the use of either FBS or SR in the culture medium [2]. Several groups have previously reported successful culturing of hESCs on HFFs and other human feeders [4–6, 9–12]. Primary HFFs are isolated from infant foreskin, which can easily be obtained on a regular basis. The level of effort for derivation and culture of HFF is low compared with MEFs, as the isolation of the primary line from a tissue sample is simple and the primary cells can be easily expanded, whereas MEFs are generally only used in passages 2–3.

Previous attempts to use ITS as a medium supplement failed due to problems with spontaneous differentiation, resulting in failure to maintain hESCs beyond passage 11 [6, 9, 15]. In

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Figure 3. Genetic characterization of the xeno-free human embryonic stem cell (hESC) line SA611 (passage 9). (A): The chromosomes from SA611 were diploid normal. The figure shows a representative karyotype. (B, C): Fluorescence in situ hybridization analysis of selected chromosomes from SA611 demonstrated that the cells were XY and diploid normal for chromosomes chromosome 12 and 17 (B) and for X (blue), Y (gold), 13 (red), 18 (aqua), and 21 (green) (C).

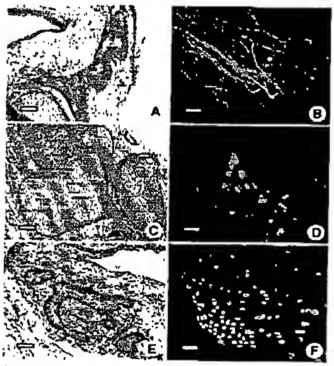

Figure 4. Confirmation of pluripotency of the xeno-free human embryonic stem cell (hESC) line SA611 in vivo (A, C, E) and in vitro (B, D, F). Histological analysis of teratomas from SA611 after 11 passages under xeno-free conditions was as follows. (A): Neuroectoderm (ectoderm); (C): Cartilage (mesoderm); (E): Secretory epithelium (endoderm). In vitro-differentiated SA611 cells were analyzed by immunofluorescence 2–4 weeks after passaging. (B): β-III-tubulin-positive neurons (ectoderm); (D): ASMA-positive smooth muscle actin (mesoderm); (F): HNF3β (Foxa2)-positive cells (endoderm). Scale bars = 50 μm (A, B, D–F), 100 μm (C).

contrast, we show that the use of HS as a medium supplement in combination with HFFs supports genetically stable (>50 passages) maintenance of self-renewal and pluripotency of hESCs.

The methods for preparing HS are not stated in any of the previous reports [6, 9, 15], but it is known that different methods of serum preparation may yield HS of different quality, with various effects on cell growth [13]. This could be an explanation for the divergent results. Alternatively, the discrepancy may be explained by the fact that we used a higher concentration of FGF (10 ng/ml) than previous attempts (4–8 ng/ml) [9, 15]. FGF is known to be an important factor in promoting hESC self-renewal [16, 17] in vitro. Having shown that xeno-free HS-supplemented medium, together with HFF feeders, supports maintenance of genetically and phenotypically stable hESCs, we next derived new HFF feeders under rigorously xeno-free conditions.

The final obstacle to overcome was the elimination of all animal components normally used during the isolation of the ICM. This procedure is traditionally performed by immunosurgery, which involves the use of rabbit polyclonal antibodies and guinea pig serum. Together with others, we recently reported [14, 18] that the immunosurgery procedure can be excluded, thus allowing ICM isolation without exposure to animal components. We chose to use acid Tyrode's solution for ICM isolation under xeno-free conditions, as it is used in IVF units for assisted hatching procedures [19] and has also been used to remove the zona pellucida prior to immunosurgery [20]. ICM isolation is also possible by mechanically opening up the zona pellucida to allow natural hatching, but that method requires the use of a micromanipulation system.

By optimizing the incubation time in acid Tyrode's solution, the zona pellucida was efficiently removed at the same time as the trophectodermal cell layer was damaged. As this treatment could not be used to destroy the trophectoderm completely without damaging the ICM, the initial outgrowth from the treated blastocysts was composed of a heterogenous cell population. However, with time, areas of morphologically distinct hESCs appeared, which could be handpicked and transferred to fresh plates. The fact that already at passage 5, colonies from line SA611 homogenously expressed Oct-4 and morphologically resembled undifferentiated hESCs, with a small cytoplasm-to-nucleus ratio, demonstrates that immunosurgery is not necessary for derivation of hESCs.

In summary, by developing alternative xeno-free procedures both for deriving and growing hESCs, we successfully established and characterized the first xeno-free hESC line, demonstrating that animal components are not necessary for the establishment and culture of hESCs. The hESC line SA611 can be used as a source of xeno-free cells for future applications in basic research and regenerative medicine. Of course, there are additional issues that should be addressed prior to clinical application of hESCs. One remaining concern is the possible introduction of unknown human pathogens to the hESC cultures by the blastocysts, feeders, or human serum. This concern is not limited to hESC culture but is to be generally considered in all allogenic transplantations in today's clinical reality. Therefore, improved pathogen screening technology, as well as more defined synthetic supplements, should be considered.



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## Disclosures

H.S. owns stock in, has acted as a consultant for, and has served as an officer or member of the board for Cellartis AB within the last 2 years.

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**Derivation of a Xeno-Free Human Embryonic Stem Cell Line**

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# Establishment and Maintenance of Human Embryonic Stem Cell Lines on Human Feeder Cells Derived from Uterine Endometrium under Serum-Free Condition[1]

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## ABSTRACT

Human embryonic stem (hES) cells are usually established and maintained on mouse embryonic fibroblast (MEFs) feeder layers. However, it is desirable to develop human feeder cells because animal feeder cells are associated with risks such as viral infection and/or pathogen transmission. In this study, we attempted to establish new hES cell lines using human uterine endometrial cells (hUECs) to prevent the risks associated with animal feeder cells and for their eventual application in cell-replacement therapy. Inner cell masses (ICMs) of cultured blastocysts were isolated by immunosurgery and then cultured on mitotically inactivated hUEC feeder layers. Cultured ICMs formed colonies by continuous proliferation and were allowed to proliferate continuously for 40, 50, and 55 passages. The established hES cell lines (Miz-hES-14, -15, and -9, respectively) exhibited typical hES cells characteristics, including continuous growth, expression of specific markers, normal karyotypes, and differentiation capacity. The hUEC feeders have the advantage that they can be used for many passages, whereas MEF feeder cells can only be used as feeder cells for a limited number of passages. The hUECs are available to establish and maintain hES cells, and the high expression of embryotrophic factors and extracellular matrices by hUECs may be important to the efficient growth of hES cells. Clinical applications require the establishment and expansion of hES cells under stable xeno-free culture systems.

*cell line establishment, embryo, human embryonic stem cell lines, human endometrium, human feeder, xeno-free culture*

## INTRODUCTION

Human embryonic stem (hES) cells were first isolated from inner cell masses (ICMs) of human blastocysts by Thomson and colleagues [1]. The hES cells exhibit unique features, including continuous growth, a high level of telomerase activity, normal karyotypes, and differentiation capacity in vivo and in vitro. They express pluripotent cell-specific markers: strongly positive for stage-specific embryonic antigen (SSEA)-3 and -4, tumor rejection antigen (TRA)-1-60 and -81, and a high level of alkaline phosphatase (APase) activity, but are negative for SSEA-1 [1–7]. They also express Oct-4, a transcription factor that is specific to ICMs of blastocysts [3, 5–8]. Continuous proliferation of ES cells requires leukemia inhibitory factor (LIF) and/or a mitotically inactivated feeder cell layer in cultures of mouse embryonic stem (mES) cells. In contrast with the culturing of mES cells, feeder cell layers and basic fibroblast growth factor (bFGF) are necessary to maintain hES cells in an undifferentiated state with or without LIF, but LIF alone cannot support the continuous proliferation of hES cells with preventing the differentiation [1, 3, 9]. The hES cells are usually established and maintained on mouse embryonic fibroblast (MEF) feeder layers. Because animal feeder cells are associated with risks, including pathogen transmission and viral infection, and ethical problems, many stem cell researchers have investigated different culture conditions for hES cells, such as a feeder-free culture system. Extracellular matrices (ECMs) and MEF-conditioned (MEF-CM) medium were introduced to expand hES cells rather than the feeder cells. The hES cells cultured under these conditions show the same characteristics as hES cells cultured on MEF feeder layers [10]. However, this culture system also requires the massive and continuous culturing of MEF cells to obtain MEF-CM medium, and the possibilities of mouse retroviral infections and pathogen transmission remain [11–13]. Attempts to prevent infections and pathogen transmission have involved the cultivation of hES cells on human feeder layers, such as fetal muscle, fetal skin, adult fallopian tubal epithelial cells [14], foreskin fibroblasts [6, 11], and adult marrow cells [15]. Additionally, Richards and colleagues [16] reported the testing of various human feeder cells derived from fetal (muscle, skin), adult (lung, skin, fallopian tube, muscle, endometrium), and neonatal (foreskin) tissues, and some of these cells are now available for use. The hES cells cultured on these various human feeder layers exhibit the same characteristics as MEF-based hES cells, including continuous proliferation, positive expression of specific markers (SSEA-3 and -4, TRA-1-60 and -81, APase, and Oct-4), normal karyotypes, and differentiation capacity. Although some feeder cells alter the shape of hES cell colonies, almost all hES cells cultured on human feeder cells maintain typical hES cell morphology, such as being round and small with a high nucleus-to-cytoplasm ratio, the notable presence of one to three nucleoli, and typical intercell spacing [6, 11–16].

Human uterine endometrial cells (hUECs) regulate embryonic development and successful implantation in the reproductive track. Their growth and differentiation vary according to the menstrual cycle due to hormonal regulation. Throughout the menstrual cycle, hUECs express various factors, including growth factors (IGF, EGF, and TGFβ)

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[17–21], cytokines (CSF, LIF, and interleukin-1 and -6) [22–27], and cell adhesion molecules (ECMs and integrin) [28–31] to control embryonic development and allow successful implantation. Some of these factors (such as LIF and TGFβ1) secreted in hUECs are known to be key regulators related to hES cell self-renewal. Additionally, various ECMs and cell adhesion molecules are expressed in hUECs, and these factors also have an important role in maintaining the undifferentiated state of hES cells [12].

In the present study, we examined the availability of hUECs as a feeder cell, attempted to establish and maintain new hES cell lines by using hUEC feeder cells, and confirmed the characteristics of newly established hES cell lines.

## MATERIALS AND METHODS

### Preparation and Culture of Human Endometrial Cells

Human endometrium was obtained by biopsy following institutional review board (IRB) approval and the receipt of informed consent from the subjects. The obtained endometrium was minced finely and digested enzymatically with 0.25% collagenase (Sigma, St. Louis, MO) and 10 U/ml DNase I (Sigma) in DMEM/F-12 (GibcoBRL/Invitrogen, Grand Island, NY). Dissociated hUECs were cultured in DMEM/F-12 supplemented with 10% FBS (Hyclone, Logan, UT), 4 mM glutamine (GibcoBRL/Invitrogen), 20 mM HEPES, 100 U/ml penicillin, and 100 μg/ml streptomycin (Sigma).

### Preparation of Feeder Layers

Cultured hUECs were mitotically inactivated with 10 μg/ml mitomycin C (Sigma) for 1.5 h and washed three times with PBS. Mitotically inactivated hUECs were then trypsinized with trypsin-EDTA (GibcoBRL/Invitrogen) and washed twice with culture medium. The dissociated hUECs were counted and plated on gelatin-coated four-well plates at $8.0 \times 10^5$ cells per well (Nunc, Roskilde, Denmark).

### Culture of Human Embryos

Cryopreserved human pronuclear (PN)-stage embryos were donated following IRB approval and the receipt of informed consent by couples undergoing in vitro-fertilization treatment. Eight rapidly thawed PN-stage embryos were first cultured in G1.2 medium until the eight-cell stage, and then transferred to G2.2 medium (Vitrolife, Denver, CO) [7, 32]. After 3–4 days of prolonged culture, seven fully expanded blastocysts were collected to isolate ICMs.

### Immunosurgery

Cultured blastocysts were first removed from the zona pellucida using 0.5% Pronase (Sigma). These blastocysts were exposed to 100% anti-human-serum antibody (Sigma) for 20 min and washed three times with PBS for 5 min. Washed blastocysts were transferred to guinea pig complement (Life Technologies, Karlsruhe, Germany) at 37°C in 5% $CO_2$ [32]. After 20 min, the blastocysts were washed and the isolated ICMs were transferred onto mitotically inactivated hUEC feeder layers.

### Culture of hES Cells

Immunosurgery was performed on seven blastocysts to obtain their ICMs. To culture isolated ICMs, the culture medium for feeder cells was changed to DMEM/F12 containing 20% knockout serum replacement, 1 mM glutamine, 0.1 mM β-mercaptoethanol, 1% nonessential amino acids, and 4 ng/ml bFGF (GibcoBRL/Invitrogen). Transferred ICMs were cultured on hUEC feeder layers for 6–8 days in the initial passage, after which the cultured cells formed colonies. These colonies were split onto newly prepared hUEC feeder layers, and the culture was prolonged. After 10 passages of the culture, the colonies were split every 5 days.

### Immunocytochemistry

After 20 passages of the culture on hUEC feeder layers, hES cells were tested for the expression of pluripotent cell-specific markers. To detect APase activity, cultured cells were fixed with 4% paraformaldehyde. Fixed cells were permeabilized with 0.2% Triton X-100 and washed three times with PBS. APase staining was performed using a kit containing NBT/BCIP as the substrate (Roche Molecular Biochemicals, Indianapolis, IN). When continuously proliferating cells appeared dark blue, we observed the stained colony under light microscopy. The expressions of surface marker antigens were confirmed using SSEA-3, -4 (positive), and -1 (negative). Fixed cells were incubated with each primary antibody and were localized with biotinylated secondary antibody conjugated with avidin and horseradish peroxidase complex (Vectastain ABC system; Vector Laboratories, Burlingame, CA). The localization, color reaction, and visualization of red staining by light microscopy were performed using a Vector NovaRED substrate kit (Vector Laboratories).

### Cell Line Identification and Karyotyping

We extracted each genomic DNA from Miz-hES-9, -14, -15, and hUEC and applied DNA fingerprinting using short tandem repeat (STR) loci amplified by PCR to identify established hES cell lines [32]. The STR loci that we used were D3S1358 (Chromosome 3p), vWA (12p12-pter), FGA (4q28), amelogenin (X:p22.1–22.3 &Y:p11.2), TH01 (11p15.5), TPOX (2p23-2per), CSF1PO (5q33.3-34), D5S818 (5p22-31), D13S317 (13q22–31) and D7S820 (7q11.21–22).

To analyze the karyotypes of hUEC feeder cells and hES cells, cell division was blocked by 0.1 μg/ml colcemid (Gibco/Invitrogen) in metaphase for 1–2 h. Cells were then trypsinized and resuspended in hypotonic KCl solution (Sigma), incubated for 20 min at 37°C, and fixed with 3:1 methanol:acetic acid. Chromosomes were visualized using G-band staining. More than 100 cells were examined in this way.

### Differentiation of hES Cells In Vivo and In Vitro

The hES cell colonies that were cultured for longer than 20 passages on hUEC feeder layers were harvested clear of feeder cells. Prepared colonies were injected with a sterile 25 gauge needle into the right testis of 4-wk-old SCID-beige mice according to the statement on the use of animals in our institute. The injected mice were killed 12 wk after this injection and the resulting tumors were fixed with 4% paraformaldehyde and embedded in paraffin. The paraffin blocks were sectioned at 10 μm, stained with hematoxylin-eosin, and the tumors observed under a light microscope.

To confirm the differentiation of hES cells in vitro, they were harvested mechanically and washed to remove the feeder cells. Harvested hES cells were transferred into embryoid body (EB) culture medium (DMEM/F-12 supplemented with 20% serum replacement, 1 mM glutamine, 0.1 mM β-mercaptoethanol, and 1% nonessential amino acids except for bFGF) and cultured continuously. At days 7, 14, and 24 after the beginning of culture, EBs were prepared for RNA isolation. Total RNA isolation from hES cells and differentiated cells was performed using TRIzol reagent according to the manufacturer's protocol (GibcoBRL/Invitrogen). Isolated RNA was quantified with a spectrophotometer (Spec3000; Bio-Rad, Hercules, CA) and reverse transcription (RT) was performed with 1 μg of total RNA in each sample. To confirm the differentiation ability of hES cells, PCR was performed with various differentiation-marker primers: ectoderm (NF-68: forward, acgctgaggaatggttcaag; reverse, tagacgcctcaatggttcc; keratin: forward, aggcccaatacgaggagatt; reverse, atagccactggagatggtgg), mesoderm (CMP: forward, aaaaagggcaatgacaccag; reverse, tgtgcagtctctgaggtgg; kallikrein: forward, gctttctcagccaggacatc; reverse, tatcttgcctccccaggtg; enolase: forward, gttcaatgtcatcaatggcg; reverse, gtgaacttctgccaagctcc), and endoderm (α-FP: forward, tgaaaaccctcttgaatgcc; reverse, tcttgcttcatcgtttgcag; α1-AT: forward, actgtcaacttcggggacac; reverse, cccattgctgaagacctta). Oct-4 (forward, gacaacaatgagaaccttcaggaga; reverse, ttctggcgccggttacagaacca) was used as a positive control for undifferentiated hES cells [3, 5–8]. PCR cycles consisted of an initial denaturation step at 94°C for 5 min, followed by 30 cycles of 30 sec of denaturation at 94°C, 30 sec of annealing at 62°C, and 30 sec of extension at 72°C. A final extension was performed at 72°C for 10 min. PCR products were visualized by ethidium bromide staining following 1.5% agarose gel electrophoresis.

### Flow Cytometry

To analyze the DNA content of hES cells, Miz-hES-9, -14, and -15 colonies were harvested with 0.1% collagenase IV (Sigma) and washed twice with PBS. These colonies were dissociated into single cells with 0.5 mM EDTA (Sigma). Separated cells were fixed with 70% ethanol at 4°C for more than 1 h and then washed clean. Fixed cells were stained with 100 μg/ml propidium iodide (Sigma) containing 100 μg/ml RNase, and measured by flow cytometry (Epics Altra; Beckman Coulter, Miami, FL).

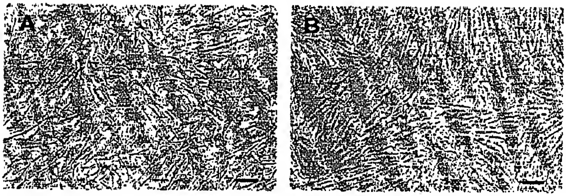



Fig. 1

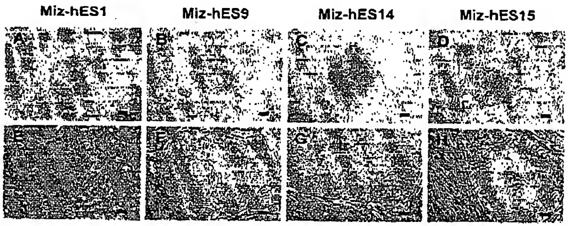



Fig. 2

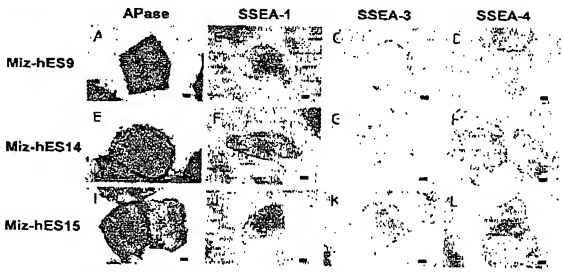



Fig. 3

PLATE I:

FIG. 1. Phase contrast photographs of MEF (A) and hUEC (B) feeder cells. They showed obviously different morphology. MEF cells had a spiky and irregular outline, and hUECs were spindle-shaped. Each feeder cell was mitotically inactivated with mitomycin C and then plated onto gelatin-coated four-well plates at $8.0 \times 10^5$ cells per well. Bar = 100 μm.

FIG. 2. The morphology of hES cell colonies expanded on MEF (A, E) or hUEC (B–D, F–H) feeder layers. Miz-hES-1 (A, E) expanded on MEF feeder layers showed typical hES cell morphology, such as being round and small with a high nucleus-to-cytoplasm ratio, the notable presence of nucleoli, and typical intercell spacing. The hUEC based Miz-hES-9 (B, F), -14 (C, G), and -15 (D, H) also showed similar morphology to Miz-hES-1. However, the shape of hUEC-based hES cell colonies were thinner, flatter, and more angular than MEF-based Miz-hES-1. Bar = 200 μm (A–D) and 100 μm (E–H).

FIG. 3. Immunocytochemical staining of undifferentiated hES cells established on hUEC feeder layers. Miz-hES-9, -14, and -15 expanded on hUEC feeder layers showed the same expression patterns of pluripotent cell-specific markers as MEF-based hES cells. The hUEC-based hES cells were positive for SSEA-3 (C, G, K) and -4 (D, H, L) and APase (A, E, I) but were negative for SSEA-1 (B, F, J). Bar = 200 μm.

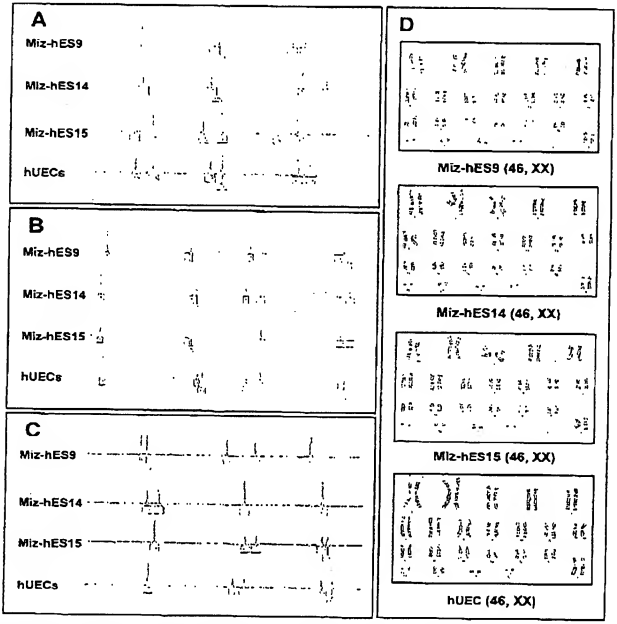



FIG. 4. DNA fingerprinting (A–C) and karyotype analysis of Miz-hES-9, -14, -15 and hUEC (D). A) Isogenic analysis in loci D3S1358, vWA, and FGA. B) Isogenic analysis in loci Amelogenin, TH01, TPOX, and CSF1PO. C) Isogenic analysis in loci D5S818, D13S317, and D7S820. The matching probabilities are $7.8 \times 10^{-11}$ for Miz-hES-9, $1.9 \times 10^{-14}$ for Miz-hES-14, $1.6 \times 10^{-14}$ for Miz-hES-15, and $3.7 \times 10^{-15}$ for hUEC. The boxed numbers and corresponding peaks represent locations of polymorphisms for each short tandem repeat marker. D) Miz-hES-9, -14, -15 and hUECs showed normal 46, XX karyotypes.

## RESULTS

### *Culture of Primary hUECs*

Three hUEC lines were isolated and cultured: two (Miz-endo1 and -2) from endometrial tissues obtained at the midproliferative phase, and the third (Miz-endo3) from the tissue obtained at the midluteal phase. Miz-endo1 and -2 could be cultured for more than 25 passages, whereas Miz-endo3 showed limited proliferation. Frozen-thawed hUECs isolated at the midproliferative phase exhibited similar growth characteristics. Miz-endo1 and -2 showed similar growth patterns, and hence we mainly used Miz-endo1 in this experiment. The morphology of Miz-endo1 cells was different from that of MEF feeder cells (Fig. 1). Miz-endo1 and -2 were split every 5 days because they reached confluence within a short period of time. At passage 3, cultured hUECs were first used as feeders to support the establishment and maintenance of hES cells and were used continuously until they became senescent.

### *Maintenance of hES Cell Lines Established on hUEC Feeder Layers*

In the culture of donated embryos, seven blastocysts were obtained from eight cultured PN-stage embryos, and the ICMs of these blastocysts were isolated by immuno-

FIG. 5. Histological analysis of teratomas formed in the testes of SCID-beige mice by the injection of Miz-hES-9 (A, B), -14 (C, D), and -15 (E, F). Well-differentiated teratomas contain kidney-like structure (A), primitive neuroepithelial tubule (B), skin (C), gastrointestinal epithelium (D), tooth-like structure (E), and cartilage (F). Bar = 100 μm.

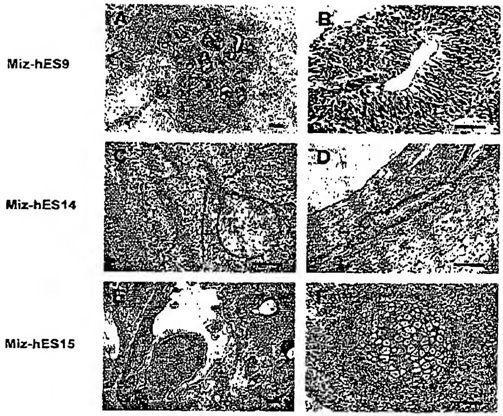
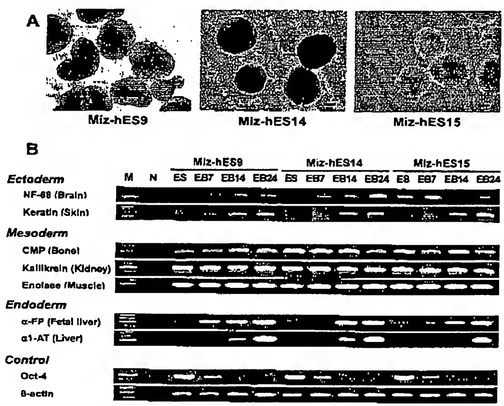



FIG. 6. In vitro differentiation of hES cells established and expanded on hUEC feeder layers. To confirm the differentiation capacity of hUEC-based hES cells, total RNA was extracted from sphere-shaped 7-, 14- (A), and 24-day-old EB, and RT-PCR was performed to examine the expression of tissue-specific markers (B). The expression of keratin (ectoderm), CMP (mesoderm), α-FP, and α1-AT (endoderm) was gradually increased in a time-dependent manner in EBs. However, NF-68, kallikrein, and enolase were expressed in both undifferentiated hES cells and differentiated EBs. The expression of Oct-4 gradually decreased in differentiated cells. Bar = 100 μm.



surgery. Three transferred ICMs were attached to hUEC feeder layers and continuously proliferated in the initial passage. Proliferating cells formed colonies after 7–8 days in the culture, and these colonies were split onto newly prepared hUEC feeder layers. They exhibited slow proliferation compared with the growth of other hES cells (Miz-hES-1, National Institutes of Health registered) until passage 7 or 8. Miz-hES-9, -14, and -15 were in continuous cultures for 55, 40, and 50 passages, respectively. They have been split onto new feeder layers every 5–6 days. The hES cells cultured on hUEC feeder layers were thinner, flatter, and more angular than MEF-based hES cells, and boundaries between hES cells and feeder cells were more evident (Fig. 2).

### *Expression of Pluripotent Cell-Specific Markers*

The expression of several cell-surface markers was confirmed by immunocytochemistry. Miz-hES-9, -14, and -15 expanded on hUEC feeder layers showed the same expression patterns of pluripotent cell-specific markers as MEF-based hES cells. The hUEC-based hES cells were positive for SSEA-3 and -4 and APase but were negative for SSEA-1 (Fig. 3). Feeder cells were negative for all surface makers used in this experiment.

### *DNA Finger Printing and Karyotype Analysis*

All hES cell lines could be distinguishable because they showed different STR loci, including D3S1358 (chromosome 3p), vWA (12p12-pter), FGA (4q28), amelogenin (X: p22.1~22.3 &Y:p11.2), TH01 (11p15.5), TPOX (2p23-2per), CSF1PO (5q33.3~34), D5S818 (5p22-31), D13S317 (13q22~31), and D7S820 (7q11.21~22) (Fig. 4, A–C).

Karyotype analysis was performed with hUECs and hES cells expanded on hUEC feeder layers. Miz-endo1, and Miz-hES-9, -14, and -15 showed normal 46, XX karyotypes (Fig. 4D), as did the frozen-thawed hUECs (data not shown). Karyotype analysis was performed every 6 mo.

### *Formation of Teratomas and EBs*

SCID-beige mice were killed 12 wk after being injected with undifferentiated hES cells to confirm the formation of teratomas. Teratomas had formed in the right testes of all three mice injected with Miz-hES-9, -14, and -15 and appeared as well-differentiated tumor-like structures, including kidney-like structure, primitive neural tube, skin, gastrointestinal epithelium, tooth-like structure, and cartilage (Fig. 5).

EBs cultured for 7, 14, and 24 days in suspension also showed the expression of tissue-specific markers. Almost all of the tissue-specific markers were expressed in differentiated EBs, with the expression level gradually increasing with time from the induction of differentiation. However NF-68, kallikrein, and enolase were also expressed in undifferentiated hES cells, and the expression of Oct-4 gradually decreased in differentiated cells (Fig. 6).

### *Proliferation Properties of hES Cells*

MEF and hUEC feeder cells comprised 10.0% and 10.24% of proliferating cells, respectively, but the composition of proliferating cells was higher in Miz-hES-9 (47.6%), -14 (48.6%), and -15 (48.2%) lines than in feeder cells. Each hES cell line established and maintained on hUEC feeder layers showed a slightly higher composition of proliferating cells than MEF-based Miz-hES-3 (40.4%).

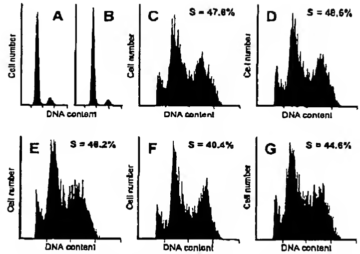



FIG. 7. Flow cytometric analysis of feeder cells and hES cells. Cultured hUECs (A), MEFs (B), and Miz-hES-9 (C), -14 (D), and -15 (E) were fixed and stained with propidium iodide. DNA content was measured by fluorescence-activated cell sorting analysis. Each feeder cell consisted of similar amounts of proliferating cells (about 10%). Proportion of Miz-hES-9 (47.6%), -14 (48.6%), and -15 (48.2%) in S phase was similar to each other. Miz-hES-3 (G, 44.6%) expanded on hUEC feeders showed slightly higher composition of proliferating cells than MEF-based Miz-hES3 (F, 40.4%).

Miz-hES-3 expanded on hUECs for more than 20 passages showed a slightly increased proportion of the cells in the S phase (44.6%; Fig. 7).

## DISCUSSION

The hES cells are usually expanded on feeder layers to maintain an undifferentiated state. MEF cells have been used as feeder layers prepared from CF-1 mice at 13.5 days after fertilization, but animal feeder layers exhibit some problems in maintaining hES cells in an undifferentiated state. First, there is an ethical problem associated with animal feeder layers because hES cells directly interact with MEF cells to maintain continuous proliferation. Second, animal feeder layers are associated with risks of viral infection and pathogen transmission. Because the ultimate object of stem cell research is cell-based clinical therapy, hES cells should be expanded on human feeder layers or under feeder-free culture conditions. There are some previous reports on this, but there are some problems that MEF cells must be cultured to obtain MEF-CM continuously and MEF-CM still has the possibility of viral infection or pathogen transmission.

In feeder-free culture conditions for hES cells, ECMs, including laminin, collagen, and fibronectin, play an important role in maintaining hES cells in an undifferentiated state. In 2001, Xu and colleagues [10] were the first to demonstrate that the expression levels of integrin$\alpha_6$ and $\beta_1$ in hES cells cultured on Matrigel or laminin were similar to those in MEF-based hES cells, and that these ECM-based hES cells maintained typical hES cell characteristics. The expression level of laminin and proliferation capacity were slightly higher in hUECs and hES cells cultured on hUECs. We thought that the expression level of laminin in feeder cells may affect the maintenance of hES cells in an undifferentiated state and that other factors expressed in feeder cells, such as LIF and TGF$\beta$1, may regulate the growth of hES cells. There are previous reports that LIF

and TGFβ are expressed in normal human endometrium and regulate downstream signaling through the activation of JAK/STAT kinase and Smads, respectively. Oct-4 and Nanog are associated with hES cell self-renewal, and the expression of these factors is regulated by LIF, BMP, and TGFβ [33–35]. The hES cells maintained on hUEC feeder cells might be regulated by their proliferation and differentiation by spontaneous expression of these factors in hUEC feeder cells.

The hUECs directly interact with embryos and regulate the embryonic development and successful implantation in vivo. Although the characteristics of these cells differed with the endometrial phase, some factors, including ECMs, growth factors, and cytokines are expressed in hUECs throughout the menstrual cycle. Richards and colleagues [16] attempted to use hUECs as feeder cells to support the growth of hES cells and reported that adult glandular endometrium and adult stromal endometrium cannot maintain hES cells in an undifferentiated state. However, we have established and maintained three hES cell lines on hUEC feeder cells in our institute. Our different results may be attributable to the different endometrial phases used. Miz-endo1 and -2 obtained from the midproliferative phase can support the continuous growth of hES cells and maintain hES cells in an undifferentiated state, whereas Miz-endo3 obtained from the midluteal phase cannot.

The hUECs have the advantage that they can be used for many passages, whereas MEF feeder cells exhibit a limited number of passages when they were used as feeders. However, hUECs showed a similar capacity to support the continuous growth of hES cells before they become senescent. An hUECs feeder cell line can be used for more than 1 yr, allowing the long-term culturing of hES cells under stable conditions. In addition to this, human feeder cells are more convenient than primary cultured MEF feeder cells in terms of the absence of a requirement for animal facilities.

For the eventual application in cell-based therapy, hES cells should be free from the risks of pathogen transmission and viral infection, and it is ideal that hES cells are cultured under stable xeno-free culture condition. Until now, however, it has been hard to eliminate animal materials completely for establishment and expansion of hES cells. Immunosurgery, a common method to isolate ICMs of blastocysts, has been performed by using anti-human-serum antibody purified from rabbit and guinea pig complement [1, 3, 6, 7]. Although these materials do damage to only trophoectoderms, there is still a possibility of exposure to animal and/or viruses. Also, animal serum (usually FBS or FCS) has been used for attachment and growth of feeder cells to support the establishment and growth of hES cells [1–3, 6, 7, 11, 14–16, 32]. Although hES cells are cultured under feeder-free condition risks of pathogen transmission and viral infection still remains because the materials from animal sources such as feeder-CM and ECM gel (usually Matrigel) have still been used for the growth of hES cells [10, 12, 13]. Up to now, there have been no hES cell lines established and maintained under complete xeno-free culture condition even though some have been represented as a xeno-free culture system for the culture of hES cells without animal serum and by preventing direct interaction with animal cells [14, 16]. In our experiment, we also partially used animal materials for immunosurgery and culture of hUECs. However, Miz-hES-9, -14, and -15 were established and maintained without direct interaction with animal cells and animal serum. Therefore, we thought that our results are in the process of becoming complete xeno-free culture conditions.

Our results demonstrate that hES cell lines can be established and maintained on hUEC feeder layers. Newly established hES cell lines have typical hES cell characteristics such as continuous growth, expression of cell surface markers, normal karyotype, and differentiation capacity. These hES cells show elevated proliferation capacity, which might be due to the effects of the expression levels of ECMs and embryotrophic factors in feeder cells (data not shown). The establishment and expansion of hES cells under a completely xeno-free culture system might be helpful to cell-based therapies. Therefore, continuous investigation for the improving culture condition should be performed to make complete xeno-free culture systems. These will lead us to obtaining available hES cells under xeno-free culture system in the near future.

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transactions are the difficulty in accessing correct and complete information on potential partners, suppliers or market possibilities and the uncertainty of ensuring a partner's commitment to formal contracts. The latter in particular, requires a judicial system that functions more efficiently and credibly. These conditions have nothing to do with TRIPS. Thus, the impact of TRIPS on either the commercial strategies of foreign companies or their strategic alliances with Indian companies is anyone's guess, as it is only one parameter among many that will be used in making foreign investment decisions.

From the perspective of Western firms, the implementation of TRIPS in India may encourage them to introduce new brand drugs because such products will now enjoy patent protection—a situation not possible since 1970. This will not mean, however, that high-priced, Western-manufactured products can be directly shoehorned into the Indian market. As K.S.N. Prasad, CEO of Shantha Biotechnics (Hyderabad, India), puts it: "Though TRIPS gives exclusive rights to Western companies to market their brand products in India—eliminating competition from local companies that copy inventions—these multinationals are unlikely to benefit from selling their products at high prices because Indian consumers simply cannot afford the high costs of drugs developed and manufactured abroad. Therefore, it will be necessary for Western and Indian companies to enter into strategic alliances so that novel drugs can be manufactured under license for local consumption. Such alliances will lead to a win-win situation for all, both biotech companies and the public."

To sum up, Indian biotech firms basically have three choices in the short-term as business innovation strategies[2]: first, they can focus on products that are either off-patent already or soon to be off-patent (essentially the generics market); second, they can collaborate with Western multinationals and biotech companies (two areas that are likely to witness an increase in collaborations are clinical trials and R&D outsourcing); or third, they can focus on innovations that the multinationals will not be interested in; that is, mainly 'tropical' or developing world diseases.

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# Preserving the genetic integrity of human embryonic stem cells

**To the editor:**

The limited number of human embryonic stem (hES) cell lines[1–7] heightens the need to maintain their genetic integrity. A report by Draper *et al.* published in last January's issue (*Nat. Biotechnol.*, 22, 53–54, 2004) and a related correspondence from Buzzard and colleagues in the April issue (*Nat. Biotechnol.* 22, 381–382) suggest that hES cell lines propagated *in vitro* for even a few months can develop an abnormal karyotype. We report here data from our laboratory that throw more light on the genetic stability of hES cell lines and its relation to how cells are maintained in culture.

In our laboratory, US National Institutes of Health (NIH, Bethesda, MD, USA)-registered hES cell lines BG01 and BG02 were propagated by manual dissection of the hES cell colonies[3] and have normal karyotypes at passages 41, 50, 62, 74 and 105. These results confirm previous observations from Buzzard and colleagues, indicating that the difficult and laborious manual passaging of hES cells will retain a stable karyotype, even after 100 passages. Faster and easier means of passaging hES cells are available, but we found that they can promote chromosomal aneuploidy, especially trisomy 12 and/or 17, in conjunction with aberrant gene expression.

Two means of disaggregating hES cell colonies into single cell suspensions for bulk hES cell passaging[1,6,8]—nonenzymatic (cell dissociation buffer, CDB) and enzymatic (collagenase/trypsin, CT)—eventually compromised the genetic integrity of the hES cell lines that had previously been passaged by manual methods. Chromosomal abnormalities dominated the BG01 hES cell cultures after as few as 23 passages after changing over to the CDB passaging method. The BG01 cell line maintained a normal karyotype for 42 manual passages but developed trisomy 12 and 17 in all cells (5 cells out of 20 analyzed contained an additional X chromosome) as early as 23 passages after changing from the manual to the nonenzymatic CDB passaging method (Supplementary Fig. 1 online). The BG02 hES cell line maintained a normal karyotype for 45 manual passages, but trisomy 17 was observed 25 passages after switching to CDB passaging. The BG02 line, which demonstrated a normal karyotype after 12 manual passages, was found to have trisomy 12, 14, 17 and an extra copy of the X chromosome in all cells (3 cells out of 20 analybed contained an extra copy of chromosome 20), when studied 56 passages after switching to the enzymatic CT passaging method (Supplementary Fig. 2 online).

Because manually passaged BG02 hES cells maintained a normal karyotype through 105 passages, we then investigated whether limited CDB or CT passaging of hES cell colonies could be used to reduce or limit changes in karyotype. Limited disaggregation of BG02 hES cell colonies resulted in normal karyotypes for 13 and 15 passages in CDB and CT treatments, respectively (Table 1). However, trisomy for chromosome 17 was observed by CDB passage 23, indicating that chromosomal abnormalities eventually arose using current enzymatic/nonenzymatic passaging methods.

Abnormal karyotype was also associated with significant changes in expression of candidate genes implicated in maintaining pluripotency[9–11] as well as of other genes related to early developmental lineage restriction. Differential gene expression analyses by real-time PCR was conducted on four BG02 hES cell groups. We tested the effect of passage number (early versus late) in both manual and enzymatic (CT) passaged cells. Gene expression of the aneuploid late CT was compared to the normal karyotype groups (early manual, late manual and early CT), and the late CT exhibited a higher expression of genes associated with pluripotency, including *POU5F1*, *SOX2*, *LEFTY2* (also

**Table 1 Results of cytogenetic and fluorescent *in situ* hybdridization (FISH) analyses of BG01 and BG02 hES cells based on passage number and method of cell dispersal**

| | Number of passages using different dispersal methods | | | | FISH chromosome 12% signal count[b,c] | | FISH chromosome 17% signal count[b,c] | |
|---|---|---|---|---|---|---|---|---|
| Cell line | Mechanical | CDB | C/T | Karyotype[a] | 2 copies | 3 copies | 2 copies | 3 copies |
| BG01 | 41 | | | 46, XY [20] | 100% (200) | | 100% (200) | |
| BG01 | 42 | 23 | | 48, XY, +12, +17 [20]/ 49, XXY, +12, +17 [5] | 11% (22) | 89% (178) | 12% (24) | 88% (176) |
| BG02 | 50 | | | 46, XY [20] | 100% (128) | | 100% (100) | |
| BG02 | 62 | | | 46, XY [9] | 100% (145) | | 100% (200) | |
| BG02 | 74 | | | 46, XY[20] | 100% (200) | | 100% (200) | |
| BG02 | 100 | | | 46,XY | 100% (200) | | 100% (200) | |
| BG02 | 105 | | | 46, XY | 100% (200) | | 100% (200) | |
| BG02 | 45 | 7 | | 46, XY [20] | 100% (200) | | 100% (200) | |
| BG02 | 45 | 13 | | 46, XY [20] | 100% (200) | | 100% (200) | |
| BG02 | 45 | 25 | | 47, XY, +17[5]/ 47, XY, +inv(17) (q11.2q21) [2] | 100% (200) | | | 100% (200) |
| BG02 | 56 | | 3 | 46, XY [9] | 100% (100) | | 100% (100) | |
| BG02 | 65 | | 15 | NA | 100% (100) | | 100% (100) | |
| BG02 | 12 | | 56 | 50, XXY, +12, +14, +17 [17]/ 51, XXY, +12, +14, +17, +20 [3] | 7.5% (15) | 92.5% (185) | 5.5% (11) | 94.5% (189) |

[a]Values in square brackets [ ] refer to total number of metaphases. [b]≤5% signal gain or loss is considered background and is not shown. When trisomy is present, apparent 'losses' due to overlapping signals is increased so that 5.5–12% losses seen above may be due to artifact or may represent low level presence of a normal cell line. NA, not analyzed. [c]Values in parentheses () refer to total number of nuclei analyzed. Details of cytogenetic methods are presented in Supplementary Methods online.

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known as *EBAF*), *GABRB3*, *GBX2* and *FGF13* (Supplementary Table 1a online). Eleven of 17 pluripotent genes were more highly expressed in late CT hES cells compared with the three chromosomally normal hES cell groups (highlighted in Supplementary Table 1a online). Analysis of early embryonic development genes (Supplementary Tables 1b–e online) also revealed a higher expression in hES cells with an abnormal karyotype (late CT) compared with the other three karyotypically normal groups. Overall, more than 70% of the genes analyzed were found to be significantly different between the abnormal karyotype late CT group and the normal early manual, late manual and early CT groups ($P < 0.05$; Supplementary Table 1 online).

Increased expression of pluripotent genes may be a consequence of *in vitro* selection for specific aneuploid hES cell populations with enhanced proliferative ability under extended bulk passaging. Our data also indicate that, in addition to increased expression of pluripotent genes, abnormal cell lines also exhibit increased expression of early differentiation genes, implying that bulk passaging compromises the normal gene expression profile of undifferentiated hES cells.

In summary, our results suggest that bulk passage methods (CT and CDB) can perpetuate aneuploid cell populations after extended passage in culture, but may be used for shorter periods (up to at least 15 passages) without compromising the karyotypes. Together with the findings of Draper *et al.* and Buzzard and colleagues on karyotypic instability in hES cells, our data indicate that bulk passaging can lead not only to abnormal karyotypes but also to quantitative differences in gene expression. Even so, it may be possible to maintain a normal karyotype in hES cells under long-term manual propagation conditions followed by limited bulk passaging in experiments requiring greater quantities of hES cells than manual passage methods, alone, can provide. In addition, our data underscore the need for simultaneous karyotypic and quantitative gene expression analyses to preserve and monitor the genetic integrity of hES cells in continuous culture.

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# Chromosomal integrity maintained in five human embryonic stem cell lines after prolonged *in vitro* culture

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## Abstract

There have been recent reports of human embryonic stem cell (hESC) lines developing chromosomal aberrations after long-term culture, indicating an unstable genomic status due to the *in vitro* milieu. This raises concern, since it would limit their use in therapeutics. In this study the chromosomal status of five well-characterized hESC lines, SA002, SA002.5, AS034.1.1, SA121 and SA461, was monitored during long-term *in vitro* culture. The criteria of defined hESCs were met by all of the five hESC lines (four diploid and one trisomic for chromosome 13). The genomes were screened for chromosomal aberrations and rearrangements using comparative genomic hybridization (CGH), interphase fluorescence *in situ* hybridization (FISH) and traditional karyotyping on several occasions while in culture. The genomic integrity was shown to be maintained after repeated freeze-thaw procedures and continuous culture *in vitro* for up to 22 months (148 passages). We discuss the most common *de novo* chromosomal aberrations reported in hESCs, as well as their possible origin.

## Introduction

Human embryonic stem cells (hESCs) have been the focus of intense research since the first successful isolation of human inner cell mass cells in 1994 (Bongso *et al.* 1994) and the establishment of the first hESC line (Thomson *et al.* 1998). The pluripotency of these cells opens the possibility for them to be used in therapeutics, for instance as a cure for Parkinson's disease and diabetes. Within the field of transplantation, hESCs may be designed to be less susceptible in terms of graft rejection within a host, compared with adult donor cells. Furthermore, these cells may be used as a tool in drug testing, as well as in developmental studies of early embryogenesis.

It has recently been suggested that hESCs are prone to acquiring chromosomal anomalies while being cultured continuously *in vitro* (Cowan *et al.* 2004, Draper *et al.* 2004, Inzunza *et al.* 2004, Maitra *et al.* 2005). The cause of such potential instability is not fully known. Iwarsson *et al.* (1999) found that frozen-thawed human embryos of good morphology had a slightly higher rate of aneuploidy than did non-cryopreserved ones. In addition, the *in vitro* culture milieu exposes the cells to several factors that may influence the genome.

In order to keep the stem cells undifferentiated they must be cultured on a feeder layer, or a supportive matrix, and in a medium containing more or less defined serum components (Amit *et al.* 2000,

Xu *et al.* 2001, Hovatta *et al.* 2003, Draper *et al.* 2004, Koivisto *et al.* 2004, Stojkovic *et al.* 2005). Propagation of the cells using mechanical dissection alone (Heins *et al.* 2004, Koivisto *et al.* 2004), or in combination with cell dissociation buffers or enzymes of different efficiency (Xu *et al.* 2001, Cowan *et al.* 2004, Stojkovic *et al.* 2005), may affect the growth conditions and possibly also the genome in different ways. One possible outcome could be additional chromosomal material (i.e. trisomy), which could subsequently affect differentiation capacity, cell cycle regulation and the growth rate of the cells. The therapeutic potential of these cells could then be questioned, suggesting the necessity to screen the stem cells for chromosomal anomalies both continuously and prior to use for such purposes. Maitra and colleagues also found changes in methylation patterns and in mitochondrial deoxyribonucleic acid (DNA) in hESCs after long-term culture (Maitra *et al.* 2005).

The most common cytogenetic tools used today are fluorescence *in situ* hybridization (FISH), karyotyping and comparative genomic hybridization (CGH). When combined, the limitations of each of these techniques, i.e. the inability to screen all the chromosomes of the genome for chromosomal changes (FISH), analytical difficulties due to the low-resolution banding pattern in stem cells (karyotyping) and the inability to detect balanced translocations, mosaicism and ploidy (CGH), may be circumvented.

This is a retrospective study comparing the chromosomal status at early and late passages in five different hESC lines. Information derived by karyotyping and FISH (for chromosomes 13, 18, 21, X and Y, in some cases also for chromosomes 12, 17 and 20) is here confirmed by the use of CGH.

## Materials and methods

### *Culture of human embryonic stem cell lines*

Surplus human embryos from clinical *in vitro* fertilization (IVF) treatments at the Sahlgrenska University Hospital in Göteborg and the Akademiska Hospital in Uppsala, Sweden, were donated after approval from the local ethics committees at Göteborg University and Uppsala University and informed consent from the donors. Human embryonic stem cell lines were established and cultured as previously described (Heins *et al.* 2004). The hESC lines were cultured on mouse embryonic fibroblasts (MEFs) and routinely passaged every 4–5 days by mechanical dissection using a stem cell tool (Swemed Lab International AB, Billdal, Sweden). The medium used was VitroHES™, manufactured by Vitrolife AB (Kungsbacka, Sweden), with addition of 4 ng/ml human recombinant basic fibroblast growth factor (hrbFGF).

The following hESC lines were used in this study: SA002, SA002.5 (a sub-line of SA002), AS034.1.1 (a sub-line of AS034.1, which is a sub-line of AS034), SA121 and SA461. The SA lines were donated by Sahlgrenska University Hospital, Göteborg, and the AS line was donated by Akademiska Hospital, Uppsala. All five cell lines have been chromosomally analysed after being frozen and thawed twice (see Table 1). The cell lines have been extensively

*Table 1.* Passage number of cell lines at chromosomal analysis and at freezing/thawing.

| Cell line | FISH | Karyotyping | CGH | Freezing/thawing |
|---|---|---|---|---|
| SA002 | 19, 23, 27, 86, 146* | 23, 25, 91 | 41 | 3, 12 |
| SA002.5[a] | +31, 134 | +19, +41 | +59 | +5, +22 |
| AS034.1.1[b] | +50 | +19, +22 | +52 | +7, +17 |
| SA121 | 35, 45, 77, 134, 148 | 35 | 77 | 24, 31 |
| SA461 | 12, 36 | 13, 24 | 36 | 2, 10 |

The five cell lines were analysed using fluorescence *in situ* hybridization (FISH), karyotyping and comparative genomic hybridization (CGH) in the passage numbers depicted in each column. Fluorescence *in situ* hybridization was performed with probes specific for chromosomes 13, 18 and 21 and the gonosomes. The "+" symbol in front of the passage number for cell line SA002.5 and AS034.1.1 indicates culture of the maternal cell line for 155 (a) and 71 (b) passages, respectively.

[a]Cell line clonally derived from SA002 at passage 155.

[b]Cell line clonally derived from AS034.1 at passage 71, which in turn is clonally derived from AS034 at passage 74.

*This FISH result is based on only 18 nuclei, all trisomic for chromosome 13 and disomic for chromosomes 18, 21 and X.

characterized, as described earlier (Heins *et al.* 2004), by expression analysis of markers characteristic for undifferentiated (Oct-4, SSEA-3, SSEA-4, TRA-1-60 and TRA-1-81) and differentiated (SSEA-1) hESCs, measurement of alkaline phosphotase (ALP) activity, telomerase activity and *in vivo* and *in vitro* pluripotency.

### *Karyotyping and fluorescence in situ hybridization*

Karyotype preparations and FISH analyses were performed at different passages of the five hESC lines, as shown in Table 1. For mitotic preparations, cells were cultured in VitroHES™ supplemented with 0.1 mg/ml colcemid (Invitrogen, Stockholm, Sweden) for up to 1 hour. The cells were harvested and fixed according to the method described by Deng *et al.* (2003). Mitotic preparations were stained with diamidino-phenylindole (DAPI) (50 ng/ml) (Vysis, Inc., Downers Grove, IL, US).

For FISH analysis, cells were fixed on glass slides as previously described (Jakobsson *et al.* 1995). A commercially available kit containing probes specific for chromosomes 13, 18, 21, X and Y was used (MultiVysion™ PGT Multicolour Probe Panel, Vysis, Inc.). Based on reported aberrations in hESCs, probes for chromosomes 12, 17 and 20 were successively included in the FISH analysis. At the time, SA461 had been frozen and hence was not analysed for chromosomes 12, 17 and 20. The other four cell lines were studied at the following passages for chromosomes 12 and 17: SA002 at passage 27; SA002.5 at passage +31 ("+" indicates passage number after clonal derivation, see Table 1); AS034.1.1 at passage +50; and SA121 at passage 45 and 134. AS034.1.1 was also studied for chromosome 20 at passage +50. All probes were purchased from Vysis, Inc. (CEP 12, CEP 17, CEP 20). Hybridization and washing of slides was performed as described elsewhere (Hardarson *et al.* 2003). Between 100 and 200 nuclei were analysed for every cell line. Slides were analysed on a fluorescence microscope equipped with appropriate filters and software (Cytovision, version 3.5; Applied Imaging, Newcastle upon Tyne, UK). As a control for normal diploid cells, lymphocytes from a karyotypically normal male were used in addition to recommended values supplied with the probes. With regard to the FISH scoring criterion, signals had to be separated by the diameter of at least one signal to be scored as two signals. The margin of error inherent in the FISH technique was taken into account for each probe, based on values supplied by the manufacturer.

Cell line SA002 (trisomic) and sub-line SA002.5 (disomic) were more frequently analysed with the use of FISH for chromosome 13 with the specific aim to evaluate the degree of mosaicism over time.

Between 10 and 20 cells were karyotyped for each cell line, except for cell line AS034.1.1 in passage 22, and cell line SA461 in passage 13, for which only six analysable metaphase plates per cell line could be found. Cell lines with a majority of diploid karyotypes and aberrant karyotypes with random losses/gains (up to 10% with the same chromosome pair involved), were considered to be normal.

### *Comparative genomic hybridization*

At the time of DNA extraction, colonies were allowed to grow for 7–8 days on MEFs before they were mechanically isolated. Blood from a karyotypically normal male was used as reference material. Genomic DNA was extracted from the five hESC lines at separate passages (see Table 1), using a salting out technique. In short, 1 × red cell lysis buffer (for 5 × buffer: 1.6 mol/L sucrose, 5% TritonX-100, 1 mol/L Tris-HCl and 25 mmol/L $MgCl_2 \times 6H_2O$ diluted to 500 ml) was added to heparinized blood and centrifuged. The pellet was treated with Proteinase K and appropriate buffer at 37°C overnight. After addition of 6 mol/L NaCl and centrifugation the DNA was precipitated by absolute ethanol. After washing in ice-cold ethanol (70%) the concentration was estimated using a spectrophotometer (GeneQuant; Pharmacia Biotech Ltd, Science

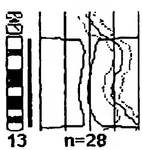


*Figure 1.* Comparative genomic hybridization ideogram profile for chromosome 13 in SA002 at passage 41. The ideogram indicates a surplus of the whole chromosomal material, shown as a green bar along the whole q-arm. N = 28 chromosomes analysed.

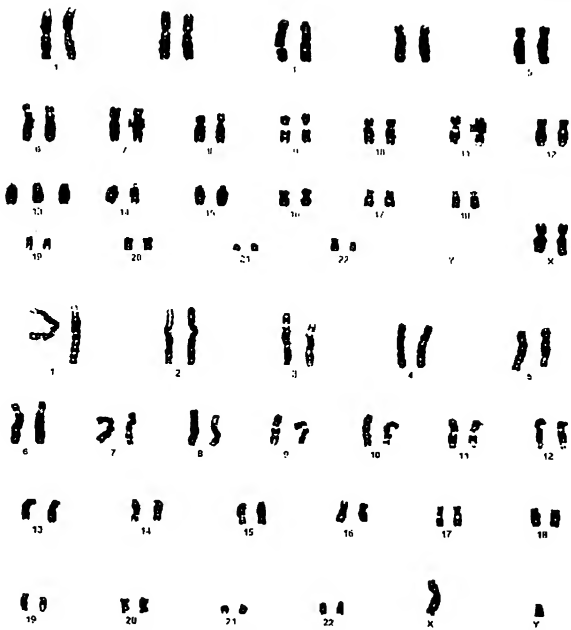

*Figure 2.* Female trisomic and male diploid karyotypes. Two representative karyotypes of SA002 (47,XX,+13) at passage 23 and of AS034.1.1 (46,XY) at passage 22.

Park, Cambridge, England) and verified by electrophoresis. As hybridization slides, normal metaphase CGH target slides (Vysis, Inc.) were used.

Labelling of DNA was performed using nick translation, according to Vysis' protocol http://www.vysis.com/CGHprocedure_33021.asp 13/9/

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*Figure 3.* Fluorescence in situ hybridization for chromosomes 13, 18, 21, X and Y in SA121 at passage 148. After two freeze-thaw procedures and *in vitro* culture for 22 months fluorescence *in situ* hybridization (FISH) analysis showed a diploid chromosomal status regarding chromosomes 13 (spectrum red), 18 (spectrum aqua), 21 (spectrum green), X (spectrum blue) and Y (spectrum gold). Scale bar indicates 5 μm.

2005). All reagents were purchased from Invitrogen (Sweden), except fluorescein-12-dUTP and Texas Red-5-dUTP (PerkinElmer Life Sciences, Inc., Boston, MA, US). After labelling, the lengths of the DNA fragments were verified by gel electrophoresis to be in the interval of 600–2000 base pairs (bp). The probe was precipitated, hybridized to target slides and washed according to Vysis' protocol.

For analysis, the CytoVision CGH software program, version 3.5, was used together with a fluorescence microscope (Nikon Eclipse E600) equipped with appropriate filters. For each cell line, ten to 15 mitotic cells passing the CGH quality control test were analysed using 99.5% confidence limit and dynamic standard reference interval thresholds, and taking into account variations in normal samples.

## Results and discussion

### *Chromosomal stability in the five human embryonic stem cell lines studied*

Of the five hESC lines analysed and previously shown to meet the criteria of a defined hESC, all but one showed a diploid genome. Cell line SA002 carried a chromosome 13 trisomy (Figures 1 and 2) detected at passage 19. However, with subsequent FISH analysis a few individual cells with two copies of chromosome 13 were observed on separate occasions. This sudden appearance of diploid cells diminished after additional passages and eventually these cells disappeared. This sporadic mosaicism provided the opportunity for deriving a diploid subline and SA002.5 was established, with two copies of chromosome 13 of unknown origin (i.e. maternally or paternally derived). It is possible that SA002 has a somewhat unstable karyotype owing to its congenital trisomy.

The other cell lines maintained genomic stability even after freeze-thaw procedures and continuous *in vitro* culture for 36–148 passages (5–22 months) (Table 1 and Figure 3). With regard to FISH analysis of chromosomes 12, 17 and 20 (20 only analysed in AS034.1.1) all four cell lines studied, SA002, SA002.5, AS034.1.1 and SA121, were diploid. A comparison of the information derived by FISH and karyotyping showed that both methods were consistent in revealing the same chromosomal status in all five cell lines. The karyotype and FISH results were further verified by CGH at later passages.

### *De novo aberrations in human embryonic stem cell lines and proliferation rate*

Human embryonic stem cells may be prone to specific genetic changes involving addition of the whole or parts of chromosomes 12, 17, 20 or X (Cowan *et al.* 2004, Draper *et al.* 2004, Inzunza *et al.* 2004, Rosler *et al.* 2004, Maitra *et al.* 2005, Mitalipova *et al.* 2005). Why these chromosomes seem to be involved more often in genetic aberrations is not yet fully clear, but it has been speculated that karyotypic changes that increase the dosage of the genes on specific chromosomes could provide a selective advantage for proliferation *in vitro* (Cowan *et al.* 2004). In the study by Cowan and colleagues, hESCs in an early passage had a longer population doubling time than did hESCs that had been in culture for longer periods of time (i.e. more than 40 passages) which also coincided with occurrence of trisomy of chromosome 12. Draper and co-workers reported a gain of chromosome 17q in two cell lines on four independent occasions (Draper *et al.* 2004). This trisomy was at first not seen in all nuclei, but the

trisomic fraction became successively larger after additional passages. By contrast, the cell lines studied in our laboratory showed a stable chromosomal constitution over time, apart from short periods of low-degree mosaicism (47,XX + 13/46,XX) in SA002. Interestingly, the spontaneously occurring disomic cells in cell line SA002 never succeeded in outgrowing the trisomic cells (data not shown), indicating that an extra chromosome 13 does not seem to negatively affect cell proliferation rate. However, the proliferation rate may also be influenced by the frequency of passaging and the density with which the hESCs are seeded.

*Passaging techniques used and culture conditions*

One possible cause of genetic changes may be the technique used when passaging the cells. Karyotypic aberrations have mostly been observed when enzymatic or chemical passaging of the stem cells has been applied (Amit *et al.* 2000, Cowan *et al.* 2004, Draper *et al.* 2004, Inzunza *et al.* 2004, Mitalipova *et al.* 2005). In addition, selection of cells to be passaged differs between chemical/enzymatic and mechanical passaging. When colonies are mechanically cut into smaller pieces, selection is based on the morphology of the cells, and colonies or areas with a differentiated morphology can therefore be avoided. Using enzymatic dissociation, however, selection of cells is based on adherence rather than morphological differences, in addition to a possible sensibility of the cells to the enzyme itself. Mechanical cutting is, however, a more time-consuming technique.

Current culturing techniques expose the cells to different factors, such as feeder layers or matrices of animal origin and serum that may give rise to genetic changes. Also, already before establishment of hESC lines the early pre-implantation embryo may be affected by the culture conditions *in vitro*, with possible subsequent influence on the stem cell genome (Gardner & Lane, 2005).

## Conclusions

In this paper we report chromosomal integrity in five hESC lines after two freeze–thaw procedures and, thereafter, continuous time in culture from 36 up to 134 passages or approximately 5–22 months. Cell lines were analysed using three different analytical tools. Mechanical passaging was used consistently, which could be one explanation why our hESC lines maintained a stable karyotype throughout the passages. We routinely used a commercially available probe kit specific for chromosomes 13, 18 and 21 and the gonosomes. However, reported *de novo* chromosomal aberrations in hESC lines indicate that a set of FISH probes specific for chromosomes 12, 17q, 20 and Xq (and possibly 13) would be more appropriate. Although FISH analysis using chromosome enumeration probes (CEPs) gives reliable results, it enables detection of only a fraction of possible aberrations. Hence, recurrent screening with FISH for the most common aberrations in hESCs needs to be complemented by karyotyping and/or CGH analysis initially, as well as after freeze-thaw procedures and after long-term *in vitro* culture. Further research needs to be carried out concerning different passaging techniques and culture conditions to better understand their effects on hESC chromosomal stability.

## Acknowledgements

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**A method for the establishment of a pluripotent human blastocyst-derived stem cell line**

**Field of the invention**

The present invention concerns a method for the establishment of a pluripotent human blastocyst-derived stem (BS) cell line, stem cells obtained by the method, differentiation of these cells into differentiated cells, the differentiated cells and the use of these differentiated cells in the preparation of medicaments. The undifferentiated pluripotent stem cells can be made to differentiate to a number of specialized cell types which can be utilized in the manufacture of medicaments for treating a number of conditions or pathologies involving degeneration of tissue e.g. of the pancreas leading to e.g. development of diabetes, or of the CNS (e.g. Alzheimer's, Parkinson's disease etc.) or degeneration of the CNS caused by e.g. stroke or physical trauma.

**Background of the invention**

A stem cell is a cell type that has a unique capacity to renew itself and to give rise to specialized or differentiated cells. Although most cells of the body, such as heart cells or skin cells, are committed to conduct a specific function, a stem cell is uncommitted, until it receives a signal to develop into a specialized cell type. What makes the stem cells unique is their proliferative capacity, combined with their ability to become specialized. For years, researchers have focused on finding ways to use stem cells to replace cells and tissues that are damaged or diseased. So far, most research has focused on two types of stem cells, embryonic and somatic stem cells. Embryonic stem cells are derived from the pre-implanted fertilized oocyte, i.e. blastocyst, whereas the somatic stem cells are present in the adult organism, e.g. within the bone marrow, epidermis and intestine. Pluripotency tests have shown that whereas the embryonic or blastocyst-derived stem cells (hereafter referred to as blastocyst-derived stem cells or BS cells) can give rise to all cells in the organism, including the germ cells, somatic stem cells have a more limited repertoire in descendent cell types.

In 1998, investigators were for the first time able to isolate BS cells from human fertilized oocytes and to grow them in culture see e.g. US 5 843 780 and in US 6 200 806.

The procedure used in the patent specifications mentioned above depends on the use of blastocysts with an intact zona pellucida. Furthermore, the method disclosed in these patents specifically use inner cell mass cells that have been isolated by immunosurgery for plating on mouse embryonic feeder cells. This method has several drawbacks, for example, it is time consuming, technically difficult and results in low yields of stem cells. Taken together, these drawbacks make it a costly method.

So far, only two articles have been published on establishment and characterization of hBS cells. This low number illustrates the unexpected problems associated with establishing these stem cells from human blastocysts. As a result very few hBS cell lines are available. The present invention describes a method for the preparation of hBS cell lines and a combination of method steps that independently will not be sufficient for deriving hBS cells but when used together they constitute the minimal requirement for successful derivation of hBS cells.

Furthermore, the present invention allows a successful derivation of hBS stem cell lines from hatched and intact blastocysts and allows for derivation of hBS cell lines after plating blastocysts onto feeder cells.

One of the difficulties with previously described methods has been to achieve an efficient attachment of the blastocysts to the feeder cells. This has resulted in low yields of end-product cells. The present invention addresses this problem.

Perhaps the most far-reaching potential application of hBS cells is the generation of cells and tissue that could be used for so-called cell therapies. Many diseases and disorders result from disruption of cellular function or destruction of tissues of the body. Today, donated organs and tissues are often used to replace ailing or destroyed tissue. Unfortunately, the number of people suffering from disorders suitable for treatment by these methods far outstrips the number of organs available for transplantation. The availability of hBS cells and the intense research on developing efficient methods for guiding these cells towards different cell fates, e.g. insulin-producing β-cells, cardiomyocytes, and dopamine-producing neurons, holds growing promise for future applications in cell-based treatment of degenerative diseases, such as diabetes, myocardial infarction and Parkinson's.

**Description of the invention**

The inventors have established a novel method for establishing a pluripotent human blastocyst-derived stem cell line from a fertilized oocyte, including propagation of the cell line in an undifferentiated state.

Thus, the present invention relates to a method for obtaining a pluripotent human blastocyst-derived stem cell line, the method comprising the steps of

i) using a fertilized oocyte optionally, having a grade 1 or 2, to obtain a blastocyst, optionally having a grade A or B,

ii) co-culturing the blastocyst with feeder cells for establishing one or more colonies of inner cell mass cells,

iii) isolating the inner cell mass cells by mechanical dissection,

iv) co-culturing of the inner cell mass cells with feeder cells to obtain a blastocyst-derived stem cell line.

v) optionally, propagation of the blastocyst-derived stem cell line.

In accordance with to the above, it is one object of the present invention to provide a method for establishing an undifferentiated human blastocyst-derived stem cell line. As a starting material for this procedure, fertilized oocytes are used. The quality of the fertilized oocytes is of importance for the quality of the resulting blastocysts.

In the method of the present invention, the establishment and evaluation of blastocysts are performed as described below. The human blastocysts in step i) of the method may be derived from frozen or fresh human *in vitro* fertilized oocytes. In the following is described a procedure for selecting suitable oocytes for use in a method according to the present invention. The present inventors have found that an important success criterion for the present method is a proper selection of oocytes. Thus, if only grade 3 oocytes are applied, the probability of obtaining a hBS cell line fulfilling the general requirements (described below) is low.

Donated fresh fertilized oocytes: On day 0 the oocyte is aspirated in Asp-100 (Vitrolife), and fertilized on day 1 in IVF-50 (Vitrolife). The fertilized oocyte is evaluated based on morphology and cell division on day 3. The following scale is used for fertilized oocyte evaluation:

Grade 1 fertilized oocyte: Even blastomers, no fragments
Grade 2 fertilized oocyte: <20% fragments
Grade 3 fertilized oocyte: >20% fragments

After evaluation on day 3, fertilized oocytes of grade 1 and 2 are either implanted or frozen for storage. Fertilized oocytes of grade 3 are transferred to ICM-2 (Vitrolife). The fertilized oocytes are further cultured for 3-5 days (i.e. day 5-7 after fertilization). The blastocysts are evaluated according to the following scale:

Grade A Blastocyst: Expanded with distinct inner cell mass (ICM) on day 6
Grade B Blastocyst: Not expanded but otherwise like grade A
Grade C Blastocyst: No visible ICM

Donated frozen fertilized oocytes: At day 2 (after fertilization) the fertilized oocytes are frozen at the 4-cell stadium using Freeze-Kit (Vitrolife). Frozen fertilized oocytes are stored in liquid nitrogen. Informed consent is obtained from the donors before the 5-year limit has passed. The fertilized oocytes are thawed using Thaw-Kit (Vitrolife), and the procedure described above is followed from day 2.

As described above, fresh fertilized oocytes are from grade 3 quality, and frozen fertilized oocytes are from grade 1 and 2. According to data obtained by the methods of the present invention, the percentage of fresh fertilized oocytes that develop into blastocysts is 19%, while 50% of the frozed fertilized oocytes develop into blastocysts. This means that the frozen fertilized oocytes are much better for obtaining blastocysts, probably due to the higher quality of the fertilized oocytes. 11% of the blastocysts derived from fresh fertilized oocytes develop into a stem cell line, while 15% of the blastocysts derived from frozen fertilized oocytes develop into a stem cell line. In summary, of the fertilized oocytes that were put into culture 2% of fresh fertilized oocytes developed into a stem cell line, and 7% of frozen fertilized oocytes that were put into culture developed into a stem cell line.

The culturing of the fertilized oocyte to the blastocyst-stage is performed after procedures well-known in the art. Procedures for preparing blastocysts may be found in Gardner et al, Embryo culture systems, In Trounson, A. O., and Gardner, D. K. (eds*), Handbook of in vitro fertilization, second edition.* CRC Press, Boca Raton, pp. 205-264; Gardner et al, *Fertil Steril*, 74, Suppl 3, O-086; Gardner et al, *Hum Reprod*, 13, 3434,3440; Gardner et al, *J Reprod Immunol*, In press; and Hooper et al, *Biol Reprod*, 62, Suppl 1, 249.

After establishment of blastocysts in step i) optionally derived from fertilized oocytes having grade 1 or 2, the blastocysts having grade A or B are co-cultured with feeder cells for establishing one or more colonies of inner cell mass cells. After being plated onto feeder cells, their growth is monitored and when the colony is large enough for manual passaging (approximately 1-2 weeks after plating), the cells may be dissected from other cell types and expanded by growth on new feeder cells. The isolation of the inner cell mass cells is performed by mechanical dissection, which may be performed by using glass capillaries as a cutting tool. The detection of the inner cell mass cells is easily performed visually by microscopy and, according, it is not necessary to use any treatment of the oocytes with enzymes and/or antibodies to impair or remove the trophectoderm.

Thus, the procedure alleviates the need for immunosurgery. By comparing the success-rate in using immunosurgery versus the present method, which leaves the trophectoderm intact, it has been observed that the much simpler, faster and non-traumatic procedure of avoiding immunosurgery is more efficient than immunosurgery. The novel procedures make the preparation of stem cell lines, and the differentiation of these cell lines commercially feasible. From a total of 122 blastocysts, 19 cell lines were established (15.5%). 42 blastocysts were processed by immunosurgery and 6 of these resulted in successfully established cell lines (14%). Eighty blastocysts were processed by the present method and 13 cell lines were established (16%).

Subsequent to dissection of the inner cell mass, the inner cell mass cells are co-cultured with feeder cells to obtain a blastocyst-derived stem (BS) cell line. After obtaining the BS cell line, the cell line is optionally propagated to expand the amount of cells. Thus, the present invention relates to a method as described above wherein the blastocyst-derived stem cell line is propagated. In one aspect, the invention relates to a method in which the propagation of blastocyst-derived stem cell line comprises passage of the stem cell line every 4-5 days. If the stem cell line is cultured longer than 4-5 days before passage, there is an increased probabilily that the cells undesirably will differentiate.

A specific procedure of passaging the cells is given in Example 5 herein.

Human BS cell lines may be isolated either from spontaneously hatched blastocysts or from expanded blastocysts with an intact zona pellucida. Thus the present invention relates to a method as described above in which the blastocyst in step i) is a spontaneously hatched blastocyst. For hatched blastocysts the trophectoderm may be left intact. Either

hatched blastocysts or blastocysts with a removed or partially removed zona pellucida may be put on inactivated feeder cells.

Zona pellucida of the blastocyst may be at least partially digested or chemically frilled prior to step ii) e.g. by treatment with one or more acidic agents such as, e.g., ZD™-10 (Vitrolife, Gothenburg, Sweden), one or more enzymes or mixture of enzymes such as pronase.

A brief pronase (Sigma) treatment of blastocysts with an intact zona pellucida results in the removal of the zona. Other types of proteases with the same or similar protease activity as pronase may also be used. The blastocysts can be plated onto said inactivated feeder cells following the pronase treatment.

In an embodiment of the invention step ii) and/or step iv) may be performed in an agent that improves the attachment of the blastocysts and/or if relevant the inner cell mass cells to the feeder cells.

A suitable substance for this purpose is a hyaluronic acid.

A suitable medium for plating the blastocysts onto feeder cells can be BS-medium that may be complemented with hyaluronic acid, which seems to promote the attachment of the blastocysts on the feeder cells and growth of the inner cell mass. Hyaluronan (HA) is an important glycosaminoglycan constituent of the extracellular matrix in joints. It appears to exert its biological effects through binding interactions with at least two cell surface receptors: CD44 and receptor for HA-mediated motility (RHAMM), and to proteins in the extracellular matrix. The positive effects of HA during the establishment of hBS cells may be exerted through its interactions with the surfactant polar heads of phospholipids in the cell membrane, to thereby stabilize the surfactant layer and thus lower the surface tension of the inner cell mass or blastocyst which may result in increased efficiency in binding to the feeder cells. Alternatively, HA may bind to its receptors on the inner cell mass or blastocyst and/or to the feeder cells and exert biological effects which positively influence the attachment and growth of the inner cell mass. According to this, other agents that may alter the surface tension of fluids, or in other ways influence the interaction between the blastocyst and feeder cells can also be used in instead of hyaluronic acid.

The inventors have also found that the culturing of the feeder cells is of importance for the establishment of the hBS cell line. In one embodiment, the propagation of blastocyst-

derived stem cell line comprises passage of the feeder cells at the most 3 times, such as e.g. at the most 2 times.

Suitable feeder cells for use in a method of the invention are embryonic feeder cells. In a method according to the invention the feeder cells employed in steps ii) and iv) are the same or different and originate from animal source such as e.g. any mammal including human, mouse, rat, monkey, hamster, frog, rabbit etc. Feeder cells from human or mouse species are preferred.

Another important criterion for obtaining an hBS cell line fulfilling the general requirements are the conditions under which the blastocysts are cultured. The blastocyst-derived stem cell line may accordingly by propagated by culturing the stem cells with feeder cells of a density of less than about 60,000 cells per $cm^2$, such as e.g. less than about 55,000 cells per $cm^2$, or less than about 50,000 cells per $cm^2$. In a specific embodiment, the propagation of blastocyst-derived stem cell line comprises culturing the stem cells with feeder cells of a density of about 45,000 cells per $cm^2$. These values apply in those cases where mouse feeder cells are used and it is contemplated that a suitable density can be found for other types of feeder cells as well. Based on the findings of the present inventors, a person skilled in the art will be able to find such suitable densities.

In a method according to the invention, the feeder cells may be mitotically inactivated in order to avoid unwanted growth of the feeder cells.

The blastocyst-derived stem cell line obtained by the present invention maintains selfrenewal and pluripotency for a suitable period of time and, accordingly it is stable for a suitable period of time. In the present context the term “stable” is intended to denote proliferation capacity in an undifferentiated state for more than 21 months when grown on mitotically inactivated embryonic feeder cells.

The stem cell line obtained by the present invention fulfils the general requirements. Thus, the cell line

i) exhibits proliferation capacity in an undifferentiated state for more than 21 months when grown on mitotically inactivated embryonic feeder cells, and
ii) exhibits normal euploid chromosomal karyotype, and
iii) maintains potential to develop into derivatives of all types of germ layers both *in vitro* and *in vivo*, and

iv) exhibits at least two of the following molecular markers OCT-4, alkaline phosphatase, the carbohydrate epitopes SSEA-3, SSEA-4, TRA 1-60, TRA 1-81, and the protein core of a keratin sulfate/chondroitin sulfate pericellular matrix proteinglycan recognized by the monoclonal antibody GCTM-2, and
v) does not exhibit molecular marker SSEA-1 or other differentiation markers, and
vi) retains its pluripotency and forms teratomas in vivo when injected into immuno-compromised mice, and
vii) is capable of differentiating.

The undifferentiated hBS cells according to the present invention is defined by the following criteria; they were isolated from human pre-implantation fertilized oocytes, i.e. blastocysts, and exhibit a proliferation capacity in an undifferentiated state when grown on mitotically inactivated feeder cells; they exhibit a normal chromosomal karyotype; they express typical markers for undifferentiated hBS cells, e.g. OCT-4, alkaline phosphatase, the carbohydrate epitopes SSEA-3, SSEA-4, TRA 1-60, TRA 1-81, and the protein core of a keratin sulfate/chondroitin sulfate pericellular matrix proteinglycan recognized by the monoclonal antibody GCTM-2, and do not show any expression of the carbohydrate epitope SSEA-1 or other differentiation markers. Furthermore, pluripotency tests *in vitro* and *in vivo* (teratomas) demonstrate differentiation into derivatives of all germ layers.

According to the above, the invention is an essentially pure preparation of pluripotent human BS cells, which i) exhibits proliferation capacity in an undifferentiated state for more than 21 months when grown on mitotically inactivated embryonic feeder cells; ii) exhibits normal euploid chromosomal karyotype; iii) maintains potential to develop into derivatives of all types of germ layers both *in vitro* and *in vivo*; iv) exhibits at least two of the following molecular markers OCT-4, alkaline phosphatase, the carbohydrate epitopes SSEA-3, SSEA-4, TRA 1-60, TRA 1-81, and the protein core of a keratin sulfate/chondroitin sulfate pericellular matrix proteinglycan recognized by the monoclonal antibody GCTM-2 v) does not exhibit molecular marker SSEA-1 or other differentiation markers, and vi) retains its pluripotency and forms teratomas in vivo when injected into immuno-compromised mice, and vii) is capable of differentiating.

Procedures for the detection of cell markers can be found in Gage, F. H., Science, 287:1433-1438 (2000). These procedures are well known for the skilled person and include methods such as RT-PCR or immunological assays where antibodies directed against the cell markers are used. In the following, methods for detection of cell markers, hybridisation methods, karyotyping, methods for measuring telomerase activity and tera-

toma formation are described. These methods can be used to investigate whether the hBS cells obtained according to the present invention fulfil the above-mentioned criteria.

*Immunohistochemistry*

The human BDP stem cells maintained in culture are routinely monitored regarding their state of differentiation. Cell surface markers used for monitoring the undifferentiated BS cells are SSEA-1, SSEA-3, SSEA-4, TRA-1-60, TRA-1-81. Human BDP stem cells are fixed in 4% PFA and subsequently permeabilized using 0.5% Triton X-100. After washing and blocking with 10% dry milk the cells are incubated with the primary antibody. After extensive washes the cell are incubated with the secondary antibody and the nuclei are visualized by DAPI staining.

*Alkaline phosphatase*

The activity of alkaline phosphatase is determined using a commercial available kit following the instructions from the manufacturer (Sigma Diagnostics).

*Oct-4 RT-PCR*

The mRNA levels for the transcription factor Oct-4 is measured using RT-PCR and gene specific primer sets (5'-CGTGAAGCTGGAGAAGGAGAAGCTG (SEQ ID NO: 1), 5'-CAAGGGCCGCAGCTTACACATGTTC (SEQ ID NO: 2)) and GAPDH as housekeeping gene (5'-ACCACAGTCCATGCCATCAC (SEQ ID NO: 3), 5'-TCCACCACCCTGTTGCTGTA (SEQ ID NO: 4)).

*Fluorescence In Situ Hybridization (FISH)*

In one round of FISH one ore more chromosomes are being selected with chromosome specific probes. This technique allows numerical genetic aberrations to be detected, if present. For this analysis CTS uses a commercially available kit containing probes for chromosome 13, 18, 21 and the sex chromosomes (X and Y) (Vysis. Inc, Downers Grove, IL, USA). For each cell line at least 200 nuclei are being analyzed. The cells are resuspended in Carnoy´s fixative and dropped on positively charged glass slides. Probe LSI 13/21 is mix with LSI hybridization buffer and added to the slide and covered with a cover slip. Probe CEP X/Y/18 is mixed with CEP hybridization buffer and added in the same way to another slide. Denaturing is performed at 70°C for 5 min followed by hybridization at 37°C in a moist chamber for 14-20h. Following a three step washing procedure the nuclei are stained with DAPI II and the slides analyzed in an invert microscope equipped with appropriate filters and software (CytoVision, Applied Imaging).

*Karyotyping*

Karyotyping allows all chromosomes to be studied in a direct way and is very informative, both numerical and larger structural aberrations can be detected. In order to detect mosaicism, at least 30 karyotypes are needed. However, this technique is both very time consuming and technically intricate. To improve the conditions for the assay the mitotic index can be raised by colcemid, a synthetic analog to colchicin and a microtubule-destabilizing agent causing the cell to arrest in metaphase, but still a large supply of cells are needed ($6x10^6$ cells/analysis). The cells are incubated in the presence of 0.1µg/ml colcemid for 1-2h, and then washed with PBS and trypsinized. The cells are collected by centrifugation at 1500rpm for 10min. The cells are fixed using ethanol and glacial acetic acid and the chromosomes are visualized by using a modified Wrights staining.

*Comparative genomic hybridization*

Comparative genomic hybridization (CGH) is complementary to karyotyping. CGH gives a higher resolution of the chromosomes and is technically less challenging. Isolated DNA is nicktranslated in a mixture of DNA, A4, Texas red -dUTP/ FITC 12-dUTP, and DNA polymerase I. An agarose gel electrophoresis is performed to control the size of resulting DNA fragments (600-2000 bp). Test and reference DNA is precipitated and resuspended in hybridization mixture containing formamide, dextrane sulfate and SSC. Hybridization is performed on denatured glass slides with metaphases for 3 days at 37°C in a moist chamber. After extensive washing one drop of antifade mounting mixture (vectashield, 0,1µg/ml DAPI II) is added and the slides covered with cover slips. Slides are subsequently evaluated under a microscope and using an image analysis system.

*Telomerase activity*

Since a high activity has been defined as a criterion for BS cells 6 the telomerase activity is measured in the BS cell lines. It is known that telomerase activity successively decrease when the cell reaches a more differentiated state. Quantifying the activity must therefore be related to earlier passages and control samples, and can be used as a tool for detecting differentiation. The method, Telomerase PCR ELISA kit (Roche) uses the internal activity of telomerase, amplifying the product by polymerase chain reaction (PCR) and detecting it with an enzyme linked immunosorbent assay (ELISA). The assay is performed according to the manufacturer's instructions. The results from this assay shows typically a high telomerase activity (>1) for BS cells.

The cell lines retain their pluripotency and forms teratomas in vivo when injected into immuno-compromised mice. In addition, in vitro these cells can form BS cell derived bodies. In both of these models, cells characteristic for all germ layers can be found.

*Teratoma formation in immunodeficient mice*

One method to analyze if a human BS cell line has remained pluripotent is to xenograft the cells to immunodeficient mice in order to obtain tumors, teratomas. Various types of tissues found in the tumor should represent all three germlayers. Reports have showed various tissues in tumors derived from xenografted immunodeficient mice, such as striated muscle, cartilage and bone (mesoderm) gut (endoderm), and neural rosettes (ectoderm). Also, large portions of the tumors consist of disorganized tissue.

Severe combined immunodeficient (SCID) -mice, a strain that lack B- and T-lymphocytes are used for analysis of teratoma formation. Human BS cells are surgically placed in either testis or under the kidney capsule. In testis or kidney, BS cells are transplanted in the range of 10 000-100 000 cells. Ideally, 5-6 mice are used for each cell line at a time. Preliminary results show that female mice are more post-operative stable than male mice and that xenografting into kidney is as effective in generating tumors as in testis. Thus, a female SCID-mouse teratoma model is preferable. Tumors are usually palpable after approximate 1 month. The mice are sacrificed after 1-4 months and tumors are dissected and fixed for either paraffin-or freeze-sectioning. The tumor tissue is subsequently analyzed by immunohistochemical methods. Specific markers for all three germlayers are used. The markers currently used are: human E-Cadherin for distinction between mouse tissue and human tumour tissue, α-smooth muscle actin (mesoderm), α –Fetoprotein (endoderm), and β-III-Tubulin (ectoderm). Additionally, hematoxylin-eosin staining is performed for general morphology.

The hBS cell line obtained by the method according to the method of the present invention can be used for the preparation of differentiated cells. Therefore the invention also relates to such differentiated cells.

In a further embodiment, the hBS cell line according to the invention has the ability of differentiating into an insulin producing cells. They may be capable of forming islet-like structures, and the amount of insulin producing β-cells is generally higher than 25%, such as e.g. higher than 35%, or higher than 40%, or higher than 45%, or higher than 50%.

Thus in one embodiment, the insulin producing cells produces at least about 300 ng insulin/mg total protein such as at least about 380 ng insulin/mg total protein or at least about 450 ng insulin/mg total protein.

The blastocyst-derived stem cells may have the ability to differentiate into differentiated cells, which display the expression of pancreatic cell type markers, including at least one of insulin, Glut-2, Pdx-1, glucokinase, glucagon and somatostatin.

Alternatively the hBS cells have the ability to differentiate into insulin-producing cells characterized by their organization into islet-like structures comprising an inner core of β-cells surrounded by an outer layer of neuron-type cells, which neuron-type cells display expression of at least one of the following neuronal cell type markers, including neuron-specific β-III tubulin (TUJ1), NeuN, DoubleCortin, tyrosine hydroxylase and Map 2.

An object of the invention is also to provide an essentially pure preparation of BS stem cells that can be made to differentiate into oligodendrocytes, and also to provide an essentially pure preparation of oligodendrocytes prepared by this method. Oligodendrocytes can be characterized by the presence of cell markers such s RIP, GalC or O4.

The blastocyst-derived stem cells that are capable of being made into differentiated cells may display the expression of at least one of the following neuronal cell type markers, including neuron-specific β-III tubulin (TUJ1), NeuN, DoubleCortin, tyrosine hydroxylase and Map 2.

In a still further aspect, the invention relates to the use of a preparation of differentiated cells derived from the blastocyst-derived stem cells obtained by a method according to the invention for the manufacture of a medicament for the prevention or treatment of pathologies or diseases caused by tissue degeneration.

A further object of the invention is to provide cells that may be used for the preparation of a medicament for treating and/or preventing diseases that may be cured by “cell genesis”. By the term “cell genesis” is meant the generation of new cells such as neurons, oligodendrocytes, schwann cells, astroglial cells, all blood cells, chondrocytes, cardiomyocytes, oligodendroglia, astroglia, and/or different types of epithelium, endothelium, liver-, kidney-, bone-, connective tissue-, lung tissue-, exocrine and endocrine gland tissue-cells.

In an embodiment, the invention relates to the use of a preparation of differentiated cells derived from the blastocyst-derived stem cells obtained for the manufacture of a medicament for the prevention or treatment of pathologies or diseases in the pancreas such as diabetes including diabetes type I.

The differentiated cells derived from the blastocyst-derived stem cell line obtained may also be used for the manufacture of a medicament for the prevention or treatment of pathologies or diseases in the nervous system. Such diseases include multiple schlerosis, spinal chord injury, encephalopathies, Parkinson's disease, Huntingdon's disease, stroke, traumatic brain injuries, hypoxia induced brain injuries, ischemia induced brain injuries, hypoglycemic brain injuries, degenerative disorders of the nervous system, brain tumors and neuropathies in the peripheral nervous system.

In a still further embodiment, the invention relates to a kit for performing the method according to the invention. The kit comprises at least a first and a second component in separate compartments. The components comprise an agent that improves the attachment of the blastocysts, a digestive agent, BS-cell medium and/or feeder cells or mixtures thereof.

The kit may further comprise blastocysts with an intact zona pelludica or spontaneously hatched blastocysts.

In another aspect, the invention relates to a method for producing an essentially pure preparation of insulin-producing differentiated stem cells, comprising the steps of;

i) expanding human blastocyst-derived stem cells by growing these on an inactivated feeder cell layer in a suitable medium;

ii) generating blastocyst-derived stem cell bodies by dissociating colonies formed in step i) into smaller aggregates or individual cells, followed by transferring said aggregates or individual cells to non-adherent containers where they are incubated in a suitable medium;

iii) plating the blastocyst-derived stem cell bodies in containers in a suitable medium;

iv) selecting nestin-positive neural precursors in ITFSn medium;

v) expanding pancreatic endocrine progenitor cells in, N2-medium comprising B27 media complement and basic fibroblast growth factor;

vi) changing the medium to a basic fibroblast growth factor-free N2 medium.

The manual dissection may be performed by using glass capillaries as a cutting tool.

The human blastocyst-derived stem cells employed in the above-mentioned method are typically those obtained as described herein.

More specifically the medium used in step i) is human blastocyst-derived stem cell medium, the medium used in step ii) is blastocyst-derived stem cell body medium, and the medium used in step iii) is blastocyst-derived stem cell body medium.

Nicotinamide may be added after step vi).

A kit according to the invention may also be applied to the above-mentioned method. In this case, the kit comprises at least two of the following components in separate compartments; mitomycin C, hBS medium, BS cell body medium, ITSFn-medium, N2-medium, B27-media supplement, nicotinamide, and bFGF.

The kit may further comprise an essentially pure human blastocyst-derinved stem cell line obtained by the method according to the present invention.

The invention is further illustrated by the following figures:

Figure 1: Blastocyst (before pronase treatment) from which human BS cell line 167 was established.
Figure 2: Blastocyst (after pronase treatment) from which human BS cell line 167 was established.
Figure 3: Blastocyst 167 two days after plating on embryonic mouse fibroblasts.
Figure 4: Human BS cells at passage 69 cultured on embryonic mouse fibroblasts.
Figure 5: Human BS cells at passage 71 cultured on embryonic mouse fibroblasts.
Figure 6: Alkaline phosphatase in BS cells (10X)
Figure 7: Alkaline phosphatase in BS cells (40X)
Figure 8: Expression of molecular markers for undifferentiated human BS cells. (A) RT-PCR analysis of total RNA extracted from undifferentiated (ud) and from differentiated (d) human BS cells for the presence of Oct-4, insulin, GLUT-2, glucagon, and PDX-1 mRNA. In controls the reverse transcriptase was omitted (-RT). β-actin serves as housekeeping gene. (B) shows the presence of alkaline phosphatase by immunostaining in undifferentiated human BS cell colonies. (C) Analysis of SSEA-1 expression by immunostaining of undifferentiated human BS cell colonies. (D) Undifferentiated BS cells were immunoposi-

tive for SSEA-3 (data not shown) and SSEA-4. (E) Immunopositive human BS cell colonies for TRA-1-60 and in (F) for TRA-1-81 showing their undifferentiated status. Magnification 40X.

Figure 9: Karyotyping of BS cells

Figure 10: Teratoma analysis: Bone

Figure 11: Teratoma analysis: Cartilage

Figure 12: Teratoma analysis: Skeletal muscle

Figure 13 Teratoma analysis: Kidney glomeruli

Figure 14: Teratoma analysis: Rosettes of neural epithelium

Figure 15: Teratoma analysis: Glandular epithelium

Figure 16: Teratoma analysis: Mucous-producing epithelium

Figure 17. Human BS cells differentiate *in vitro* into all germ layer cell types. Corresponding fluorescent micrographs show immunopositive cells stained with germ layer specific markers after 10 days in vitro. (A and B) show examples of neuroectodermal cells expressing nestin for neuronal precursors(A) and β-III-tubulin for postmitotic neurons (B) while (C) shows examples of mesodermal cells immunoreactive for Desmin; (D) examples of cells expressing α-fetoprotein.

Figure 18. Immuno staining for nestin in *in vitro* differentiated human BS cells.

Figure 19. Immuno staining for insulin in *in vitro* differentiated human BS cells.

Figure 20. Immuno staining for β-III-tubulin in *in vitro* differentiated human BS cells.

**Definitions and abbreviations**

As used herein, the term “blastocyst-derived stem cell” is denoted BS cell, and the human form is termed “hBS cells”.

As used herein, the term “blastocyst-derived stem cell bodies” is denoted “BS cell bodies”.

As used herein, the term “EF cells” means “embryonic fibroblast feeder”. These cells could be derived from any mammal, such as mouse or human.

One suitable medium used in the invention is termed “BS-cell medium” or “BS-medium” and may be comprised of; KNOCKOUT® Dulbecco’s Modified Eagle’s Medium, supplemented with 20% KNOCKOUT® Serum replacement and the following constituents at their respective final concentrations: 50 units/ml penicillin, 50 μg/ml streptomycin, 0,1 mM

non-essential amino acids, 2 mM L-glutamine, 100 μM β-mercaptoethanol, 4 ng/ml human recombinant bFGF (basic fibroblast growth factor).

Another suitable medium for the present invention is "BS cell body medium", this may be comprised as follows; KNOCKOUT® Dulbecco's Modified Eagle's Medium, supplemented with 20% KNOCKOUT® Serum replacement and the following constituents at their respective final concentrations: 50 units/ml penicillin, 50 μg/ml streptomycin, 0,1 mM non-essential amino acids, 2 mM L-glutamine and 100 μM β-mercaptoethanol (Itskovitz-Eldor, J. et al., 2000).

In the present context the term "stable" is intended to denote proliferation capacity in an undifferentiated state for more than 21 months when grown on mitotically inactivated embryonic feeder cells.

The invention will now be described with reference to the following examples. The examples are included herein for illustrative purposes only and are not intended to limit the scope of the invention in any way. The general methods described herein are well known to a person skilled in the art and all reagents and buffers are readily available, either commercially or easily prepared according to well-established protocols in the hands of a person skilled in the art. All incubations were in 37°C, under a $CO_2$ atmosphere.

**Examples**

**Example 1**

**Establishment of an essentially pure preparation of undifferentiated stem cells from spontaneously hatched blastocysts**

Human blastocysts were derived from frozen or fresh human in vitro fertilized embryos. Spontaneously hatched blastocysts were put directly on feeder cells (EF) in BS cell medium (KNOCKOUT Dulbecco's Modified Eagle's Medium, supplemented with 20% KNOCKOUT Serum replacement, and the following constituents at the final concentrations: 50 units/ml penicillin, 50 μg/ml streptomycin, 0.1 mM non-essential amino acids, 2mM L-glutamine, 100 μM β-mercaptoethanol, 4ng/ml human recombinant bFGF (basic fibroblast growth factor), supplemented with 0.125 mg/ml hyaluronic acid. After plating the blastocysts on the EF cells, growth was monitored and when the colony was large enough

for manual passaging approximately 1-2 weeks after plating) the inner cell mass cells were dissected from other cell types and expanded by growth on new EF cells.

**Example 2**

**Establishment of an essentially pure preparation of undifferentiated stem cells from blastocysts with an intact zona pellucida**

For blastocysts with an intact zona pellucida, a brief pronase (10 U/ml, Sigma) incubation in rS2 (ICM-2) medium (Vitrolife, Gothenburg, Sweden) was used to digest the zona, after which the blastocyst was put directly on the EF cell layer in BS medium supplemented with hyaluronic acid (0.125 mg/ml).

**Example 3**

**Histo-chemical staining for alkaline phosphatase**

The cells were harvested for RT-PCR and histological (alkaline phosphatase) and immunocytochemical analysis (see below).

RNA isolation and RT-PCR. Total cellular RNA was prepared using Rneasy Mini Kit (Qiagen) according to the manufacturer's recommendations. The cDNA synthesis was carried out using AMV First Strand cDNA Synthesis Kit for RT-PCR (Roche) and PCR using Platinum Taq DNA Polymerase (Invitrogen). Histochemical staining for alkaline phosphatase was carried out using commercially available kit (Sigma) following the manufacturer's recommendations.

**Example 4**

**Preparation and culturing of hBS cell line**

Mouse embryonic fibroblasts feeder cells were cultivated on tissue culture dishes in EMFI-medium: DMEM (Dulbecco's Modified Eagle's Medium), supplemented with 10% FCS (Fetal Calf Serum), 0,1 μM β-mercaptoehanol, 50 units/ml penicillin, 50 μg/ml streptomycin and 2 mM L-glutamine (GibcoBRL). The feeder cells were mitotically inactivated with Mitomycin C (10 μg/ml, 3 hrs). Human BS cell-colonies were expanded by manual dissection onto inactivated mouse embryonic fibroblasts feeder cells.

Human BS cells were cultured on mitotically inactivated mouse embryonic fibroblasts feeder cells in tissue culture dishes with BS-cell medium: KNOCKOUT® Dulbecco's Modified Eagle's Medium, supplemented with 20% KNOCKOUT® Serum replacement and the following constituents at their respective final concentrations: 50 units/ml penicillin, 50 μg/ml streptomycin, 0,1 mM non-essential amino acids, 2mM L-glutamine, 100 μM β-mercaptoethanol, 4 ng/ml human recombinant bFGF (basic fibroblast growth factor). Seven days after passage the colonies were large enough to generate BS cell bodies.

BS cell colonies were cut with glass capillaries into 0.4x0.4 mm pieces and plated on non-adherent bacterial culture dishes containing BS cell body medium: KNOCKOUT® Dulbecco's Modified Eagle's Medium, supplemented with 20% KNOCKOUT® Serum replacement and the following constituents at their respective final concentrations: 50 units/ml penicillin, 50 μg/ml streptomycin, 0,1 mM non-essential amino acids, 2 mM L-glutamine and 100μM β-mercaptoethanol (Itskovitz-Eldor, J. et al., 2000). The BS cell bodies, including cystic BS cell bodies, formed over a 7-9-day period.

**Example 5**
**Passage of hBS cells**

Before passage the hBS cells are photographed using a Nikon Eclipse TE2000-U inverted microscope (10X objective) and a DXM 1200 digital camera. Colonies are passaged every 4-5 days. The colonies are big enough to be passaged when they can be cut in pieces (0.1-0.3 x 0.1-0.3 mm). The first time the cells are passaged, they have grown for 1-2 weeks and can be cut in approximately four pieces.

The colonies are focused, one by one, in a stereo-microscope and cut in a checkered pattern according to the size above. Only the inner homogeneous structure is passaged. Each square of the colony is removed with the knife, aspirated into a capillary and placed on new feeder cells (with the maximum age of 4 days). 10-16 squares are placed evenly in every new IVF-dish. The dishes are left five to ten minutes so the cells can adhere to the new feeder and then placed in an incubator. The hBS medium is changed three times a week. If the colonies are passaged, medium is changed twice that particular week. Normally a "half change" is made, which means that only half the medium is aspirated and replaced with the equal amount of fresh, tempered medium. If necessary the entire volume of medium can be changed.

**Example 6**

**Vitrification of hBS cells**

Colonies with the appropriate undifferentiated morphology from the cell line are cut as for passage. 100-200 ml liquid nitrogen is sterile filtered into a sufficient amount of cryotubes. Two solutions A and B are prepared (A: 800 µl Cryo PBS with 1M Trehalose, 100 µl etylen glycole and 100 µl DMSO, B: 600 µl Cryo PBS with 1M Trehalose, 200 µl etylen glycole and 200 µl DMSO) and the colonies are placed in A for 1 minute and in B for 25 seconds. Closed straws are used to store the frozen colonies. After the colonies have been transferred to a straw, it is immediately placed in a cryotube with sterile filtered nitrogen.

**Example 7**

**Seeding of embryonic mouse feeder (EMFi) cells**

The cells are inactivated with EMFi medium containing Mitomycin C by incubation at 37°C for 3 hours. IVF-dishes are coated with gelatin. The medium is aspirated and the cells washed with PBS. PBS is replaced with trypsin to detach the cells. After incubation, the trypsin activity is stopped with EMFi medium. The cells are then collected by centrifugation, diluted 1:5 in EMFi medium, and counted in a Bürker chamber. The cells are diluted to a final concentration of 170K cells/ml EMFi medium. The gelatin in the IVF-dishes is replaced with 1 ml cell suspension and placed in an incubator. EMFi medium is changed the day after the seeding.

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